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THE UNIVERSITY OF ALBERTA

THE EFFECTS OF UREA- AND GLYCEROL-REMOVAL TREATMENTS ON EXCITATION-CONTRACTION COUPLING IN FROG SKELETAL MUSCLE

by

© Reinder C. Treffers

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The effects of urea- and glycerol-removal treatments on excitation-contraction coupling in frog skeletal muscle" submitted by Reinder C. Treffers in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

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ABSTRACT

Exposing frog's skeletal muscle to 400 mM urea for one hour followed by reimmersion of the muscle in a urea-free Ringer's solution (i.e. 'urea-removal treatment') abolishes the coupling between the action potential and the muscle twitch. In contrast to a similar treatment with glycerol (i.e. 'glycerol-removal treatment') the majority of the T-tubules are still freely communicating with the extracellular space. It was demonstrated that the block of excitation-contraction (e-c) coupling induced in urea-removal treated muscles was due mainly to an increase in the width of the triad junction, although some detubulation may also have occurred. Placing the muscles in Ringer's with an elevated calcium concentration (5 mM) following exposure to the hypertonic urea-Ringer's solution prevented the block of e-c coupling but not the increase in triad junctional width. These results suggest the possibility that increasing the width of the triad junction decreases the amount of 'trigger' calcium ions reaching the terminal cisternae during an action potential, thereby causing a block in e-c coupling. Elevating the extracellular calcium concentration would enhance the passive influx of calcium into muscle fibres. The resulting increase in myoplasmic free calcium would in turn increase the total amount of calcium interacting with the terminal cisternae during an action potential and thus oppose the effects of a widening of the triad junction on the e-c coupling process.

A 60 minute exposure to a hypertonic glycerol solution followed by a washout in normal Ringer's solution (glycerol-removal

treatment) also abolishes or greatly reduces e-c coupling in skeletal muscle fibres. This effect has generally been attributed to a closure of the transverse tubules at their surface openings. When muscles were subjected to a short (10 min glycerol-exposure) glycerol-removal treatment the twitch response was reduced to the same extent but measurements of the late after potential indicate that the T-system was still communicating with the extracellular space. These results imply that the closure of the T-system from the extracellular space may not have been the main cause for the lesion in e-c coupling following a (60 min) glycerol-removal treatment.

An attempt was made to elucidate the nature of the e-c coupling impairment in skeletal muscles subjected to a short glycerol-removal treatment. Unlike the effects of a urea-removal treatment, no increase in the width of the triad junction was detected. Consistent with these results was the finding that high calcium did not antagonize the reduction in e-c coupling. Electronmicroscopic observations have indicated that the main cause for the e-c coupling impairment following a short glycerol-removal treatment may be a partial breakdown of the continuity of the T-tubular lumen and subsequent structural rearrangements in the triads resulting from osmotically induced swelling and vacuolization of the T-system.

The disruption of e-c coupling in muscles subjected to a (60 min) glycerol-removal treatment is likely to be caused by a closure of the transverse tubules at their surface opening followed by the osmotically induced rupture of the T-tubular membranes.

The author of this manuscript wishes to acknowledge Dr. G.B. Frank for his able guidance and generous support in the course of this investigation, Donna Wilson for her typing and her constructive literary criticism, Gus Duchon for his patiently given advice in electron microscopic techniques, and Zuzana Forgac and Marie Novodvorsky for making my stay in the laboratory as pleasant as it was.

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CHAPTER I. INTRODUCTION

1. General Comment

Excitation-contraction (e-c) coupling is a term used to designate the processes that are involved in the coupling of electrical excitation of a muscle with its subsequent mechanical response. These processes are defined as a series of successive steps that are depicted schematically in Figure 1. It is well established (Huxley and Taylor, 1958) that excitatory stimuli, i.e. an action potential or depolarization, at the surface membrane of the twitch muscle fibres, are conducted into the interior of the fibres along the transverse tubular system. It is equally well established (Bianchi and Shanes, 1959; Winegrad, 1970) that an action potential or depolarization will cause a translocation of calcium from the sarcoplasmic reticulum to the myofilaments, resulting in muscle activation. However, the mechanism by which the electrical events at the transverse tubular level are coupled with the release of calcium from the sarcoplasmic reticulum (Figure 1, step 3) is not yet fully understood. Regardless of the exact nature of this mechanism it is evident that the triad junction which forms the anatomical link between the transverse tubules and the sarcoplasmic reticulum, must be intimately involved in this particular step of e-c coupling.

A mechanism implicating calcium ions as the coupling link between depolarization and contraction in skeletal muscle (Sandow, 1952) was first investigated by Frank (1958) and became known later as the trigger calcium hypothesis (Bianchi and Bolton, 1967; Bianchi, 1968; 1969). Other hypotheses have, and are still being considered

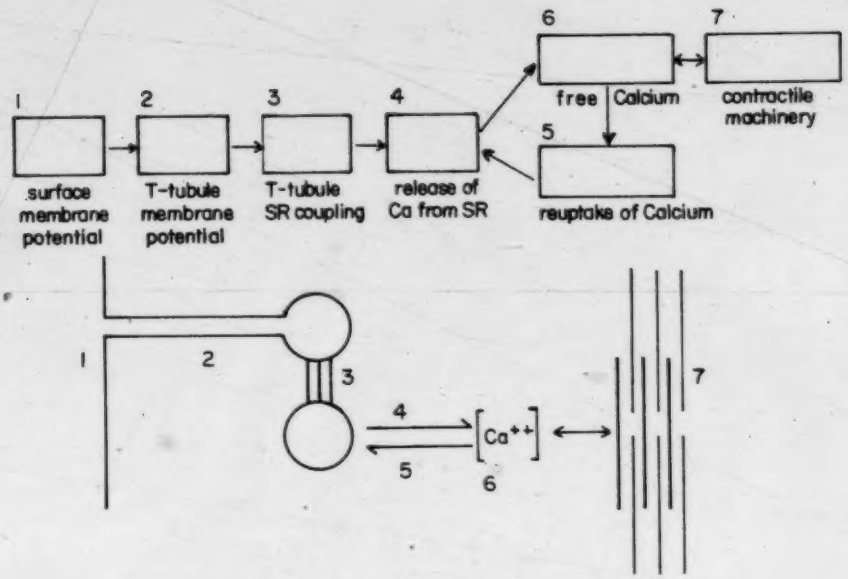


Figure 1. Schematic representation of the different steps involved in e-c coupling. Excitatory stimuli at the surface membrane (1) are conducted into the interior of the fibres along the transverse tubular system (2) and will cause the release of calcium from the sarcoplasmic reticulum (4). The released calcium (6) will activate the contractile machinery (7) and is subsequently taken up by the sarcoplasmic reticulum (5). Exactly how depolarization of the tubular membrane causes the release of calcium from the sarcoplasmic reticulum is not known (step 3).
From Morgan and Bryant (1977).

as possible candidates for a coupling mechanism (section 3).

2. Developments in E-C Coupling

Our knowledge of the different processes involved in e-c coupling has increased greatly over the past 20 years. This can be attributed to a number of outstanding experimental observations and in particular to the elucidation of the ultrastructure of the internal membrane system of skeletal muscle. The transverse tubular system, the triad junction and the sarcoplasmic reticulum have now all been recognized as separate anatomical sites along which different steps of the e-c coupling process take place. In the following sections an attempt will be made to briefly discuss the merits of the individual experimental observations as they pertain to a specific step in the overall e-c coupling process.

2.1 Transverse Tubular (T) System. One of the most significant developments in the field of e-c coupling was the observation by Huxley and Taylor (1958) that the T-system represented the morphological pathway of inwardly conducted electrical activity. These workers produced highly localized depolarizations by applying an electrical current to a pipette whose tip contacted the muscle fibre. Only when the pipette was positioned at specific sites along the sarcomere were they able to elicit a local contraction and it was found that the location of these specific sites could be correlated with the position of the T-system along the sarcomeres of the myofibrils.

The interpretation of their observations was greatly aided by the advance of the electron microscope which made it possible to describe the internal membrane system as a system of tubular and vesicular organelles (Bennet, 1955). The term triad was first coined by Porter and Palade (1957) who observed a regular repeating pattern of these organelles near the Z-lines of fast skeletal muscle fibres. A few years later Andersson-Cedergren (1959) demonstrated that the triads were composed of morphologically distinct structures; transverse tubules flanked by two lateral sacs which were part of the sarcoplasmic reticulum. Since that time it has further been shown that the T-system in amphibian muscle is an invagination of the fibre surface membrane and remains in continuity with it. Although examination of frog skeletal muscle fibres by electron microscopy usually fails to provide direct morphological evidence that the tubules are opened at the periphery of the fibre (Franzini-Armstrong et al., 1975) evidence obtained from extracellular marker studies (Endo, 1964; Huxley, 1964; Eisenberg and Eisenberg, 1968) show clearly that different marker molecules will enter the tubular network, thus confirming earlier findings by Huxley and Taylor (1958). The paucity of observed openings of the transverse tubules at the surface membrane may in part be explained by the fact that the tubular network is sparsely connected to the periphery of the fibre (Figure 2). The apparent wide circumferential separation of active spots (Huxley and Taylor, 1958) supports this notion. The infrequent detection of such openings may also be attributed to the fact that the tubules take a very tortuous course near the periphery of the fibre (Franzini-Armstrong et al., 1975) and

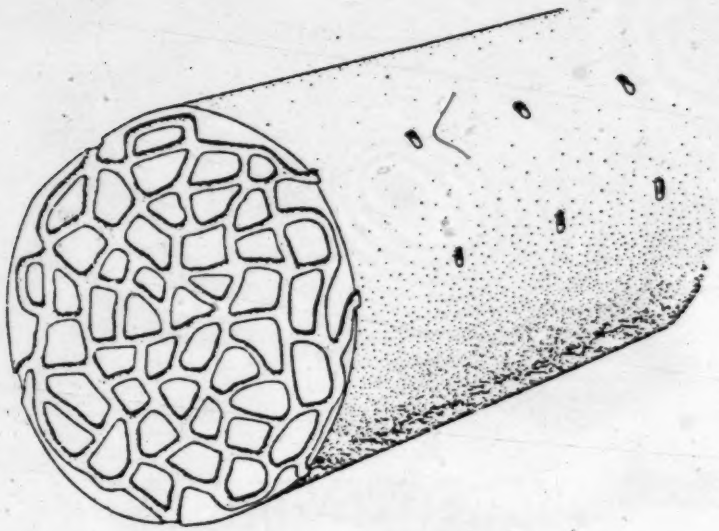


Figure 2. Illustration of the scarcity of the openings of the T-system at the surface membrane of a muscle fibre.

that these openings are identical to the many membrane-bound vesicles or caveoli (Zampighi et al., 1975). As a consequence of the continuity between the sarcolemma and the tubular membrane there is a considerable increase in surface area of the muscle fibre. For instance, Peachey (1965) estimated that for a fibre 100 μm in diameter the total surface area would be augmented seven times and that the volume of the T-system would be 0.3% of the total fibre volume. These properties of the T-system play an important part in the bioelectric behavior of the muscle and will be discussed in more detail later (section 5). The main structural features of the T-system may be summarized in Figure 3 which represents a three dimensional reconstruction of the internal membrane system and in Figure 2 which amplifies the continuity of the tubular network with the extracellular space.

Parallel to morphological studies of the T-system have been studies of its electrical activity and role in e-c coupling. Especially the mechanism of inward conduction of the electrical activity has attracted the attention of many workers. In general there are two possible ways by which an electrical signal is conducted into the centre of a muscle fibre. Firstly, an action potential confined to the sarcolemma may spread into the transverse tubular system in a purely electrotonic fashion, as proposed by several investigators (Falk and Fatt, 1964; Falk, 1968; Adrian et al., 1969). Secondly, results of a study by Gonzales-Serratos (1966) on the temperature dependence of the radial spread of activation in single muscle fibres, were more compatible with active propagation within the T-system. This was substantiated by the work of Constantin (1970) who showed

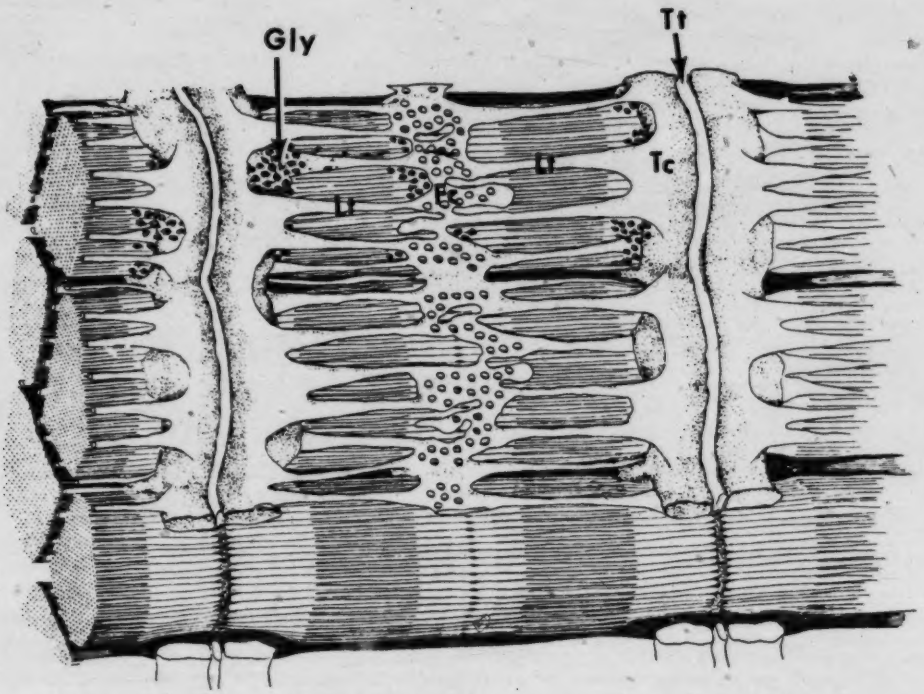


Figure 3. A three-dimensional reconstruction of the internal membrane system in association with the individual myofibrils of a muscle fibre.
From Peachey (1965).
Gly, glycogen granules; Tt, transverse tubule; Lt, longitudinal tubules; Fc, fenestrated collar; Tc, terminal cisterna.

that a surface membrane depolarization only slightly greater than the contraction threshold could produce shortening throughout the entire cross-section of a muscle fibre. He also demonstrated that the radial spread of contraction could be reduced either by lowering the extracellular sodium concentration or by the addition of tetrodotoxin (TTX) to the bathing medium. The most logical explanation for these results is that depolarization of the muscle fibre can produce an increase in sodium conductance in the transverse tubular membrane and that the resultant inward sodium current contributes to the spread of depolarization along the T-system. Subsequent studies by Gonzales-Serratos (1971) confirmed preliminary accounts of a regenerative process along the transverse tubules (Gonzales-Serratos, 1966). Microscopic observation of straightening of the individual myofibrils within an isolated muscle fibre was made possible by setting a fibre in gelatine and compressing it longitudinally. The time course of shortening of the myofibrils both near the surface and in the centre of the fibre was recorded by high-speed ciné micrography and from this measurements of the velocity of inward activation were made. The speed of inward transmission was estimated to be about 7 cm/sec at 20° C, with a Q_{10} of 2.13. The Q_{10} value is similar to that for the conduction velocity of a muscle action potential (Eccles et al., 1941) and Gonzales-Serratos (1971) concluded that it was unlikely that such a high Q_{10} would be given by a passive electrotonic system. The involvement of sodium in the excitatory process travelling along the T-system is also indicated in the experiments of Bezanilla et al. (1972). This group argued that if tubular transmission is electrotonic, the extent

of radial activation should be a function of action potential magnitude or external Na^+ concentration, whereas if the transmission process is regenerative, activation should be independent of action potential magnitude, provided sufficient Na^+ would be present to support an action potential. Their results indicate that tetanic tension of fibres in low [Na^+] solutions drops during tetanus while a similar drop is not observed in normal Ringer's solutions. They concluded that these findings were consistent with the existence of a regenerative sodium conductance in the tubular membrane during the inward spread of electrical activity. Further evidence implicating tubular sodium in the spread of inward activation was presented by Caputo and Dipolo (1973) who measured the speed of recovery of twitch tension in single fibres, following sudden changes in external [Na^+]. The recovery of twitch tension, when fibres were exposed from a sodium-free solution to one containing 46 mM Na^+, occurred more slowly in high viscosity media. The slow recovery of the twitch could be explained as a delay in diffusion of sodium into the T-system caused by the high viscosity medium and indicates the involvement of sodium in the spread of inward activation. Using a double sucrose gap method, Bastian and Nakajima (1974) applied simulated action potentials to muscle fibres treated with TTX and compared their contractile responses with responses obtained from real action potentials in untreated fibres. If the inward spread of electrical activity were passive, no significant difference should have been observed between the two methods. However the twitch height was three times larger when it was elicited by means of a real action potential. Again the evidence presented strongly suggests that the transverse tubular system is excitable and is

necessary for full activation of the twitch response. In conclusion, the experimental evidence presented thus far supports the idea that the inward transmission of excitation into the T-system of skeletal muscle fibres occurs through action potential generation rather than by passive electrotonic spread. The fundamental question of how action potentials in the transverse tubules may influence the permeability of the sarcoplasmic reticulum to calcium ions has not yet been resolved, although different theories have been hypothesized (section 3).

2.2 Sarcoplasmic Reticulum (SR) and Calcium Regulation.

The sarcoplasmic reticulum is an internal membrane system that surrounds the myofibrils. It consists of flattened sacs which extend from Z-line to Z-line. The configuration of the SR varies along the length of the sarcomere, appearing as terminal cisternae in the region of the I-band and as longitudinal tubules and a fenestrated collar in the A-band region (Figure 3). The SR appears to be discontinuous with the extracellular space of the muscle fibre although there are reports in the literature (Birks and Davey, 1969, 1972; Rubio and Sperelakis, 1972; Kulczycky and Mainwood, 1972) that favor the extracellular nature of the SR. In 1969, Birks and Davey observed that the SR of frog skeletal muscle swells when the normal bathing solution of the fibres was made hypertonic by the addition of sucrose or extra NaCl and suggested that the swelling of the SR was caused by the free diffusion of these small molecules with their osmotic equivalent of water into the SR. An earlier report based of electron microscopic data (Birks, 1965) had already described bridge-like structures between the membranes

of the T-system and SR in the region of the triad junction. These results were interpreted to suggest that the SR was an extracellular compartment which communicated with the exterior through aqueous channels (i.e. bridge-like structures) located in the triadic region (Birks and Davey 1969, 1972). From extracellular space determinations it is known that the measured space varies with the choice of the specific marker molecule (Tasker et al., 1959; Page, 1962; Bozler, 1967) and that these variations in extracellular space are merely a reflection of the degree of intracellular leakage of the different marker molecules. For instance sucrose (Tasker et al., 1959; Bozler, 1967) with a molecular diameter of 4-5 Å (Schultz and Solomon, 1961) is not confined to the extracellular space only. Efflux studies with radiosodium from whole muscles (Rogus and Zierler, 1973) indicate that NaCl is washed out from the intracellular space at two rates: a fast one corresponding to the efflux from the SR and a slower rate corresponding to its efflux from the sarcoplasm. An alternative explanation of the swelling of the SR observed in hypertonic solutions of sucrose or NaCl (Birks and Davey, 1969) would therefore be that these small MW compounds slowly leak intracellularly, preferentially into the SR.

Recent evidence from freeze fracture studies (Franzini-Armstrong, 1974) and earlier morphological observations (Franzini-Armstrong, 1970) rule out the possibility that the SR is freely accessible from the extracellular space through aqueous pores located in the triad junction (see also section 3.1). In addition electron microscopic studies with extracellular marker molecules such as Ferritin (Huxley, 1964; Page, 1964; Peachey and Schild, 1968), Thorium

dioxide (Birks, 1965) and the fluorescent dyes (Endo, 1966) have demonstrated that these molecules remain restructed to the tubular space only. Claims of other investigators (Rubio and Sperelakis, 1972; Kulczycky and Mainwood, 1972) that Horseradish peroxidase (HRP) will penetrate into the terminal cisternae and longitudinal tubules of the SR are denied by the careful study of Eisenberg and Eisenberg (1968). More consistent with the view that the extracellular space is confined to the T-system and not the SR are the experimental results obtained from a number of electrophysiological studies (sections 3.1 and 5). In these studies volume estimates of the extracellular space were extrapolated from the different electrical parameters and fell within 0.2% to 0.5% of the total fibre volume. This value agrees well with the histologically determined volume of the transverse tubular system (0.3%), but is roughly thirty times smaller than the fractional volume of the SR (Peachey, 1965). These electrophysiological studies together with the observation that diffusion of large electron-dense molecules is restricted to the T-system only, strongly support the contention that the SR is excluded from the extracellular space of skeletal muscle.

The main function of the SR is to regulate the availability of intracellular calcium to the myofilaments for muscle contraction and relaxation. The mechanism by which calcium causes contractile activation has been worked out in great detail (Ebashi and Endo, 1968; Ebashi et al., 1969; Ebashi, 1974). In short, calcium ions released from the SR diffuse into the myoplasm and bind to the calcium-receptive protein troponin, which together with tropomyosin is located along the actin filament. The association of calcium with the troponin molecule

causes a configurational change in the troponin-tropomyosin-actin complex resulting in the removal of tropomyosin's inhibition of actomyosin ATPase and enabling the contractile reaction to start. This process is rapidly reversed by the removal of calcium from the troponin molecule.

Evidence obtained from a number of biochemical studies, described below, indicate that active uptake of calcium ions into the SR is responsible for the relaxation process. The original experiments carried out by Marsh (1951) and Bendall (1953) established that a muscle extract could induce relaxation of either the actomyosin system or glycerinated fibres. This muscle extract was named the 'relaxing factor' and studies of Kumagai et al. (1955) proved it to be identical to an earlier discovered ATPase fraction (Kielley and Meyerhof, 1948 a, b). It was subsequently discovered that relaxation of glycerinated fibres could also be mimmicked by calcium chelating agents (Bozler, 1954; Ebashi, 1960) and that the relaxing factor could accumulate calcium ions in the presence of ATP (Ebashi, 1960; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961). In addition electron microscopic studies showed that the relaxing factor was composed of fragmented SR (Nagai et al., 1960; Muscatello et al., 1961; Ebashi and Lipmann, 1962). The exact details of the calcium uptake mechanism of the SR have not been worked out yet, but two general models have been proposed. Weber et al. (1966) suggested that calcium is actively transported into the lumen of the fragmented sarcoplasmic reticulum (FSR) where it is bound to a low affinity binding protein calsequestrin (MacLennan and Wong, 1971). An alternative model (Ebashi and Endo, 1968) proposes that calcium ions are first bound to sites of high calcium

affinity in the fragmented SR membrane and only part of the bound calcium is subsequently transported into the lumen of the SR. Further studies will have to establish which of the two models is the correct one.

Contrary to calcium uptake processes, the molecular mechanism by which calcium ions are released from the SR has not yet been elucidated. It is known however that a variety of different stimuli will cause the release of calcium from the SR. For instance the contractile effects induced by caffeine are mediated through calcium release from the SR (Bianchi, 1961; Weber and Herz, 1968). Other drugs such as thymol (Ebashi, 1965), quinine (Isaccson et al., 1970), halothane (Endo et al., 1975), and the free base form of certain local anaesthetics (Bianchi, 1968, 1975) were shown to have a similar action to that of caffeine. The ionophores X-537A and A23187 when added to SR vesicles loaded with calcium were shown to cause an immediate release of calcium (Scarpa and Inesi, 1972; Entman et al., 1972). A phenomenon referred to as the reversal of the calcium pump (Barlogie et al., 1971; Makinose, 1971; Makinose and Hasselbach, 1971) operates in SR vesicles at low external calcium concentrations and can induce calcium release when ADP and inorganic phosphate are added to the medium. Nakamaru and Schwarz (1972) observed calcium release form SR vesicles when they raised the pH of the medium to 8. Magnesium withdrawal has been shown to cause transient contractures in skinned fibres (Ford and Podolsky, 1972b). When the ambient temperature of intact fibres is suddenly lowered in the presence of subthreshold concentrations of caffeine, a contracture results (Sakai and Kurihara, 1974). Exposure of skinned fibres (Endo and Thorens, 1975) or

isolated SR vesicles (Kasai and Miyamoto, 1976a, b) to hypotonic media will induce calcium release. Lännergren and Noth (1973) observed contractures in intact muscles bathed in hypertonic media and attributed these to calcium release from the SR. The different stimuli that were shown to release calcium from the SR are not likely to play a role in the natural functioning of muscle contraction although some of them (i.e. caffeine, caffeine-like drugs, rapid cooling and magnesium withdrawal) may enhance the natural calcium-release mechanism (Endo, 1977).

Based on the responsiveness of the skinned fibre preparation to electrical stimulation or a changed ionic environment, Constantin and Podolsky (1967) suggested that the signal for calcium release from the SR was a depolarization of the SR membrane. Their findings were substantiated by results obtained from optical studies in intact muscle fibres (Baylor and Oetliker, 1975; Bezanilla and Horowicz, 1975). Baylor and Oetliker (1975) detected transient changes in birefringence following electrical stimulation of single muscle fibres. The birefringence signal consisted of 3 components: the first component was attributed to the surface membrane potential, the second component was thought to be related to the voltage change across the SR and associated with the release of calcium, and the third component may have reflected some conformational change in the actomyosin system. Using a different optical technique, Bezanilla and Horowicz (1975) observed a fluorescence change upon electrical stimulation of muscles stained with Nile Blue A. The fluorescence change followed the same time course as the second component of the birefringence signal (Baylor and Oetliker, 1975) and the onset of both signals occurred in the

falling phase of the action potential, some 3-6 msec before the onset of the contractile response. These results were consistent with the speculation that the optical signals reflect a SR potential change associated with the release of calcium. The SR potential change was estimated to be around 135 mV (Baylor and Oetliker, 1975) which implies that calcium is released from the total SR membrane area. Depolarization induced release of calcium was also demonstrated in fragmented SR (Kasai and Miyamoto, 1976 a, b; Inesi and Malan, 1976) but it has been suggested (Meissner and McKinley, 1976) that osmotic effects and not the membrane potential change of the SR was the real cause of calcium release. Using an intracellular electrode recording technique Strickholm (1974) detected small potential changes (1-2 mV) in muscle fibres preceeding contraction and suggested that these potential changes originated from the SR. Natori (1975) recorded propagated potential changes in contracting skinned muscle fibres. These 'internal action potentials' had a magnitude of 10 mV and were recorded with glass microelectrodes inserted into the skinned fibre. Although these microelectrode studies support the idea that SR depolarization is the signal for calcium release, the morphological observation (Peachey, 1975) that the T-system takes a spiral course around the axis of the whole fibre would seem to suggest that the electrical events associated with the SR are in fact propagated potential changes in the transverse tubular system. Moreover, the depolarization in skinned fibres (Natori, 1975) might have originated from sealed portions of the T-system in which a potential gradient had been reestablished (Nakajima and Endo, 1973). The demonstration (Winegrad, 1965, 1968, 1970) that calcium release is confined to the terminal

cisternae of the SR conflicts with results from birefringence studies (Baylor and Oetliker, 1975) which imply that calcium for contractile activation is released from the total membrane area of the SR. But by far the most compelling arguments against a depolarization-induced calcium release mechanism operating in intact fibres are: firstly, the discontinuity of the surface membrane with the membrane system of the SR (Franzini-Armstrong, 1970, 1974; sections 2.3 and 3.1), and secondly electrophysiological evidence (Ebashi and Endo, 1968; Constantin, 1975) which rules that the current flow through the triad junction is too small to cause a significant depolarization of the SR membranes.

Experimental evidence supporting a role for calcium-induced release of calcium from the SR was presented simultaneously by two groups of workers: the study by Endo's group (Endo et al., 1970) revealed that a concentration of 10^{-6} M free calcium could induce a contraction of skinned fibres. A similar response was demonstrated by Ford and Podolsky (1970). The above experimental data is compatible with the idea (Bianchi, 1969; section 3.3) that small amounts of calcium originating from the inner aspect of the transverse tubular membranes and the tubular lumen can trigger the release of additional calcium from the SR. A great deal of supportive evidence for such a calcium-induced calcium release was obtained from experiments using the skinned fibre preparation. This preparation was first used by Natori in 1954 and consists of a single muscle fibre, its sarcolemma removed mechanically and placed in an aqueous medium designed to mimmick the intracellular environment. By varying the calcium ion concentration in the medium, their effects on the release of SR calcium could be studied. Calcium release from the SR in skinned

fibres has been inferred in most cases from the occurrence of the contractile response, but has also been demonstrated directly with a variety of techniques: radioactive tracer methods (Ford and Podolsky, 1972a; Stephenson, 1975), methods employing the calcium-sensitive bioluminescent protein aequorin (Endo and Blinks, 1973) or the calcium-binding dye murexide (Endo et al., 1970). Apart from confirming the earlier findings (Endo et al., 1970; Ford and Podolsky, 1970) subsequent skinned fibre studies have helped to specify the conditions under which the calcium-induced calcium release occurs. It was shown that the minimum effective concentration of free calcium required to induce the release of SR calcium depends on the concentration of free magnesium in the medium (Ford and Podolsky, 1972b). Confirming this, Endo (1975 a, b) found that with 'physiological' magnesium concentrations (0.9 mM), a Ca^{++} concentration of 3×10^{-4} M or greater is required for calcium-release to occur. He further questioned the physiological significance of the calcium-induced calcium release, since the calculated increase in myoplasmic free calcium during an action potential in intact fibres (Curtis, 1966) was considerably lower than 3×10^{-4} M. On the other hand, it is still possible that such high concentrations of free calcium are momentarily attained in the restricted region of the triad junction during the influx of 'trigger calcium' associated with a depolarization of the transverse tubular membrane. Also, the 'physiological' free magnesium concentration (0.9 mM) may have been overestimated (Gilbert, 1960; Stephenson and Podolsky, 1977), resulting in erroneously high values for the free $[Ca^{++}]$ required to activate the release of SR calcium in skinned fibres. There is always the possibility that mechanical removal of the surface membrane will

damage the integrity of the internal membrane system and reduce the sensitivity of the calcium-release mechanism in skinned fibres. It would follow that under these conditions a higher concentration of free calcium is required to trigger the release of SR calcium. Studies with isolated SR vesicles have demonstrated that also in this preparation free calcium is able to induce the release of bound calcium into the medium (Inesi and Malan, 1976). Although the conditions under which calcium-induced release of calcium takes place have been studied extensively, little is known about the molecular mechanism of this process. It is assumed that the release of calcium results from a changed permeability of the SR membrane, triggered in some way by appropriate concentrations of free myoplasmic calcium (Endo, 1977). In conclusion, there is ample experimental evidence to suggest the operation of two separate calcium-release mechanisms *in vitro*: the *depolarization*-induced calcium release and the *calcium*-induced calcium release. The evidence presented so far seems to favour the *calcium*-induced calcium release as the mechanism that operates in intact muscle fibres *in vivo*.

2.3 *Triad Junction*. The fine structure of the triad junction has been considered extensively in the literature (Birks, 1965; Fahrenbach, 1965; Peachey, 1965; Walker and Schrodt, 1966; Kelly, 1969) but the most accurate description has been provided by the work of Franzini-Armstrong (1970, 1971, 1972, 1973, 1974, 1975). The triad junction is the area where the transverse tubular membrane and the membrane of the SR are separated by a narrow junctional gap of approximately 100 Å. At about 300 Å intervals the SR forms small projections whose tips come to within 50 Å of the transverse tubular membrane and are joined to it by some unknown amorphous material. The SR projections

and the amorphous material are normally referred to as feet. In amphibian fast skeletal muscle they are dispersed in two parallel rows on either side of the T-tubules. These junctional feet cover about 30% of the T-system surface area and 3% of the total SR area. The junctional gap represents the region across which the least understood step of the e-c coupling process takes place (Figure 1, step 3). Several attempts have been made to explain the nature of this e-c coupling step on the basis of the ultrastructure of the triad junction (section 3).

3. Theories of E-C Coupling

Three possible mechanisms whereby the potential across the walls of the T-tubules may influence the permeability of the SR membrane have been postulated. One is the existence of a low resistance pathway through which current may flow between the lumina of the SR and T-tubules. A second hypothesis proposes that a voltage dependent charge movement is responsible for the regulation of calcium permeability in the SR. The third mechanism involves the entrance of extracellular or membrane-bound calcium into the muscle fibre during depolarization of the T-system membranes and is usually referred to as the trigger calcium hypothesis.

3.1 Direct Current Flow.

Birks (1965) observed cross-bridges between the two membrane systems in the triad region and suggested that these would electrically couple the SR-T system. Fahrenbach (1965) stated that the whole triadic junction may be interpreted as a tight junction while Peachey (1965) compared the triad gap with the regularly spaced interconnections of the septate junction as it occurs

among epithelial cells. Still other workers (Walker and Schrodt, 1966; Kelly, 1969) observed membrane-like densities which were dispersed in two parallel rows on either side of the T-tubules. The densities were evenly spaced at regular intervals along the transverse tubular system and the distance between the SR and the tubular membranes was estimated to be around 100 Å. The morphological observations described above have led to the hypothesis that these interjunctional structures or densities represent the low resistance pathway which would permit membrane depolarization to progress from the T-system to the SR. However, caution is required with the interpretation of these membrane-like densities, since the dimensions of these structures are close to the practical resolution limits of the electron microscope and this fact may very well account for the great variety of observations made. Realizing these shortcomings Franzini-Armstrong conducted EM studies using differential staining techniques (1970) and freeze fracture (1974, 1975). She showed that the interjunctional structures were not an integral part of the SR-T membrane system, but consisted of amorphous material deposited between the tubular membrane and the projections of the SR (Franzini-Armstrong, 1970). Examination of the number and arrangement of particles and pits on the exposed fracture faces of T-tubular and SR membranes in the triad junction showed a lack of regular disposition in contrast to the regular hexagonal array of the feet of the triad junction (Franzini-Armstrong, 1974, 1975). In this respect the junctional membranes at the triad strikingly differ from those forming low-resistance junctions, i.e. gap junctions in epithelial cells, heart, smooth muscle and in electrotonic synapses. At all these junctions the regular array of pits and particles on the exposed

fracture faces of the junctional membranes matches the disposition of the structures that occupy the junctional gap so that the existence of continuous hydrophilic channels can be postulated (Perrachia, 1973 a, b). In view of the results obtained by freeze fracture in skeletal muscle, Franzini-Armstrong (1975) concluded that direct electrical coupling between membranes of the SR-T system was an unlikely possibility.

Evidence of a more indirect nature is also consistent with the results obtained from morphological studies: a number of electrophysiological studies have failed to demonstrate that the SR membranes provide a pathway for current flow from the sarcoplasm to the extracellular space. These studies are of two general types. (1) studies of potassium-ion accumulation and depletion within the internal membrane, and (2) studies of the effective membrane capacity of the muscle cell.

(1) Electrophysiological studies concerning the accumulation and depletion of potassium-ions in the internal membrane system as they relate to estimation of intra and extracellular spaces have been mentioned briefly in section 2.2 and will be discussed in more detail in section 5. The experimental evidence obtained from these studies indicates that the T-system has rapid access to the extracellular space and that the SR is contained intracellularly. It should therefore follow that the T-system and the SR are separated by a diffusion barrier which is inconsistent with the hypothesis that the tubular lumen and the SR are electrically coupled by cross-bridges that function as aqueous pores (Birks, 1965).

(2) The capacity of biological membranes appears to be 1 $\mu F/cm^2$ of

membrane (Curtis and Cole, 1938). The effective membrane capacity of frog skeletal muscle fibres is approximately 8 $\mu F/cm^2$ (Fatt and Katz, 1951) and is just the value that would be expected from the surface area of the T-system (Peachey, 1965). If the SR membrane had been electrically coupled with the transverse tubular membrane, as was proposed earlier, the value of the effective membrane capacity of skeletal muscle fibres would have to be considerably larger than 8 $\mu F/cm^2$.

3.2 Voltage Dependent Charge Movements. It has been proposed (Schneider and Chandler, 1973) that in skeletal muscle contractile activation is controlled by a voltage dependent charge movement similar to the gating concept in squid axons (Armstrong and Benzanilla, 1973). Schneider and Chandler were able to detect small transient currents of capacitive origin that could be associated with the movement of a definable amount of charge. They suggested that these gating currents reflected the displacement within the T-tubules of charged molecules that control the release of calcium from the sarcoplasmic reticulum. Subsequent studies by Chandler and co-workers (1967 a, b) have provided additional evidence relating these gating currents to contractile activation.

From their experimental data Chandler et al. (1976a) were able to calculate the number of fixed charge groups that were located in the T-tubular membrane and found that the density of these charged groups was the same as the density of the SR feet (Franzini-Armstrong, 1970). These workers (Chandler et al., 1976b) subsequently claimed that under resting conditions there exists some mechanical linkage

connecting both charged groups and SR feet on a one to one basis and that during activation this mechanical SR-T link could be momentarily broken resulting in the release of calcium from the SR. It is clear that this model is highly speculative and that the exact nature of the mechanical link between charged groups and the SR would have to be elucidated in future studies. In view of earlier conclusions (section 3.1) the presence of a mechanical link extending all across the triad junction is a rather unlikely possibility. Although the number of charged groups in the tubular membrane may be the same as the number of SR feet, the disposition of these charged groups does not correspond to the location of the SR feet (Franzini-Armstrong, 1975). These and other anomalies (Almers, 1975) would have to be explained before the involvement of these voltage-dependent charge movements in e-c coupling could be seriously considered.

3.3 The Trigger Calcium Hypothesis. As early as 1883 Ringer demonstrated that calcium plays an essential role in the process of muscle contraction. He showed that the frog heart failed to contract in the absence of calcium ions in the perfusion fluid. Other workers observed that under these conditions electrical activity still persisted (Locke and Rosenheim, 1907; Mines, 1913) and that the strength of contraction was proportional to the calcium concentration in the bathing medium (Bay, 1933). These findings in heart muscle led Sandow (1952) to suggest a general hypothesis concerning the role of extracellular calcium in e-c coupling. He proposed that a depolarization or action potential along the muscle fibre promoted the entrance of calcium ions into the fibre and that these calcium ions would

subsequently initiate muscle contraction. The only direct evidence supporting Sandow's hypothesis for skeletal muscle at that time was the demonstration by Heilbrunn and Wiercinski (1947) that calcium was the only physiologically occurring cation that would cause shortening when injected into bits of skeletal muscle fibre. Also in agreement with this hypothesis was the later reported influx of calcium during muscle activity (Bianchi and Shanes, 1959) and the findings by Frank (1958) who showed that removal of calcium from the bathing medium of skeletal muscle abolished its mechanical response without affecting the electrical properties.

Although the experimental evidence presented so far seemed to agree with Sandow's hypothesis, some objections were raised against a direct involvement of extracellular calcium ions in skeletal muscle activation. Firstly Hill (1949) calculated that diffusion of calcium ions from the surface membrane into the interior of the muscle during an action potential was too slow to account for the rapidity of contractile activation. Secondly, measurements of calcium influx showed that the entry per twitch was roughly one hundred times less than the amount required for full activation (Bianchi and Shanes, 1959; Frank, 1961; Curtis, 1966).

Hill's objection was overcome by the discovery that surface membrane depolarization was conducted into the interior of the muscle fibre via the T-system (Huxley and Taylor, 1958). This system reduces the diffusion path of calcium ions at the excitable membrane to the contractile elements such that its diffusion time falls well within the limits of the latent period (Frank, 1965). The involvement of calcium from a source other than extracellular or membrane-bound

calcium would have to be postulated to support the calcium concentrations required for full activation, as calculated from the calcium flux studies above. Evidence supporting the existence of another calcium source was presented by Frank (1960, 1962) who showed that caffeine-induced contractures could be obtained in calcium-free solutions at a time when potassium-induced contractures had been completely eliminated. Bianchi (1961) subsequently showed that caffeine releases calcium ions from muscles incubated in a calcium-free medium. Thus these results were consistent with the concept that calcium was contained in an intracellular store, later to be identified as the sarcoplasmic reticulum (see section 2.2). In agreement with earlier evidence (Frank, 1958) calcium involved in muscle contraction can be divided into 'membrane' calcium, associated with depolarization of the transverse tubular membrane and 'bound' calcium located in the sarcoplasmic reticulum (Frank, 1965). Bianchi (1967, 1968, 1969) was the first to assign a functional role to the different calcium stores. He postulated that depolarization of the tubular membrane causes an influx of 'membrane' calcium which diffuses across the triad gap to the terminal cisternae where it triggers the release of 'bound' calcium from the SR. Consistent with the trigger calcium hypothesis were results obtained from different studies. Two groups of workers (Endo et al., 1970; Ford and Podolsky, 1970; section 2.2) observed that the release of Ca^{++} from the SR of skinned fibres could be induced by raising the free $[Ca^{++}]$ in the medium to a certain threshold concentration.

An interesting observation that a number of multi-valent

cations were able to restore potassium-induced contractures when added to calcium-free solutions was made by Frank (1962). These cations were effective in restoring the K^+ contractures only if a store of caffeine-releasable or 'bound' calcium was still present. There is evidence to suggest that some of these multi-valent ions exert their action by binding to a membrane site (Bianchi, 1975). The implications of these findings and some related observations from our own work will be discussed later. In general these experimental results are consistent with the idea that at least some of these multivalent cations may prevent the loss of tubular membrane-bound calcium into the T-tubules so that calcium would still be available for excitation-contraction coupling when extracellular calcium is depleted.

At times the trigger calcium hypothesis has been challenged (Curtis, 1963; Edman and Grieve, 1964; Armstrong et al., 1972) These investigators claimed that the eventual failure of the twitch response in calcium-free solutions resulted from a reduction in the resting membrane potential of the muscle fibres. It was also observed that following calcium removal the twitch was maintained for some time. On the basis of these results it was concluded that extracellular calcium plays no essential role in e-c coupling. However the possibility still exists that their Ca^{++}-free solutions were contaminated by calcium from other sources; a concentration of 0.01 mM Ca^{++} ions in solution (Frank, 1960) is sufficient to support a mechanical response. Furthermore, a source of membrane-bound calcium (Weiss, 1970; Putney and Bianchi, 1974; Oota et al., 1976) less affected by Ca^{++}-removal or EGTA complexing may still be present and subserve

the function trigger calcium under these conditions. The failure to abolish the twitch response in virtually Ca^{++}-free surroundings may also be related to the choice of buffer in the physiological solution. It has been noted that phosphate-buffered muscle fibres, like the ones used by Armstrong et al. are resistant to twitch loss after removal of extracellular calcium (Frank 1978b). Evidence has been presented that the reported fall in membrane potential (Curtis, 1963; Edman and Grieve, 1964; Armstrong et al., 1972) is probably an artifact caused by the insertion of microelectrodes in membranes exposed to calcium free solutions (Frank and Inoue, 1973).

4. Hypertonicity and E-C Coupling

It has been established for quite some time that hypertonic solutions can decrease the contractile response in frog skeletal muscle (Overton, 1902) without greatly affecting the electrical properties of the individual muscle fibres (Hodgkin and Horowicz, 1957). The loss of twitch response induced by hypertonic solutions such as sucrose and NaCl is a reversible process. These molecules exert their action mainly by virtue of their relative impermeability to the muscle fibre membranes, which will give rise to an osmotic gradient and a consequent high intracellular ionic strength.

Physiological solutions made hypertonic by the addition of substances which are able to penetrate the membranes, i.e. glycerol and urea were found to affect excitatory and contractile properties of skeletal muscle in a completely different fashion (Fujino et al., 1961; Yamaguchi et al., 1962). For this reason it is necessary to distinguish between hypertonic solutions of the 'non-penetrating' (i.e. NaCl,

sucrose) and the 'penetrating' type.

4.1 Hypertonic Solutions of the Non-penetrating Type. The reduction in contractile tension in hypertonic solutions had originally been attributed to a decrease in shortening velocity of the contractile proteins (Howarth, 1958; Podolski and Sugi, 1967) and was thought to be a reflection of a decreased actomyosin ATP-ase activity of the myofilaments (Weber and Herz, 1963) under conditions of high ionic strength. April et al. (1968) confirmed that an increase in ionic strength rather than a decrease of intracellular volume was the causative factor involved in the reduction of contractile response under hypertonic conditions. Experimental results obtained by Gordon and Godt (1970) suggested that at high tonicities some disruption of e-c coupling took place in addition to the direct inhibition of the contractile filaments. In support of this hypothesis was the observation by Homsher et al.. (1974) that the activation heat, which is associated with e-c coupling, was decreased. Measurements of the active state of skeletal muscle indicate that the reduction of the twitch response in Ringer's solution made hypertonic with sucrose may have been a consequence of a diminished release of activating calcium from the sarcoplasmic reticulum (Andersson, 1973). It is possible that high intracellular ionic strength will cause structural changes in the SR membrane system and thus interfere with its ability to regulate calcium required for contractile activation. The impairment of SR-calcium regulation under hypertonic conditions may well have attributed to the observed initial release of calcium from intracellular stores (Isaacson, 1969) and consequent phasic contractures (Lännergren

and Noth, 1973).

Thus it seems that the major effect of hypertonic solutions of the 'non-penetrating' type on skeletal muscle results from a direct inhibition of myofilament movement induced by an increase in intracellular ionic strength. In addition a defect in e-c coupling becomes apparent at tonicities of 2 times normal or higher. The initial phasic contracture in hypertonic sucrose results from an enhanced release of calcium from the sarcoplasmic reticulum (Lännergren and Noth, 1973).

4.2 Hypertonic Solutions of the Penetrating Type. The loss of twitch response of muscles incubated in hypertonic glycerol (Howell and Jenden, 1967; Howell, 1969) or urea (Oota and Nagai, 1973) was found to be transient; the ability to twitch recovers during the incubation period. When the muscle is subsequently placed in an isotonic Ringer's solution the twitch response disappears while action potentials can still be generated. This procedure will be referred to as the glycerol- or urea-removal treatment.

Caffeine is still able to elicit a contracture in glycerol- or urea-removal treated muscle fibres (Howell, 1969; Oota and Nagai, 1973). This would indicate that the loss of twitch response under these conditions is not caused by a direct inhibition of the actomyosin system, but occurs in an earlier step in e-c coupling. In the case of glycerol-removal treated muscles it was suggested that the interruption of e-c coupling was associated with a general disruption of the transverse tubular system (Howell and Jenden, 1967; Gage and Eisenberg, 1967, 1969b). These findings were later confirmed in electron microscope (EM) studies which showed that extracellular markers such as

Ferritin (Nakajima et al., 1969; Krolenko, 1969) and Horseradish peroxidase (Eisenberg and Eisenberg, 1968) could not penetrate the T-system of glycerol-removal treated muscle fibres. More recent experimental evidence however, has revealed that the transverse tubules are merely constricted at their openings with the surface membrane (Nakajima et al., 1973) and it was suggested that the loss of e-c coupling in glycerol-removal treated muscle could be attributed to an effect on the triad junction instead (Dulhunty and Gage, 1973a).

In the case of urea-removal treated skeletal muscle there is evidence supporting the hypothesis that the blockade of e-c coupling is caused by an increase in the width of the triad junction (Oota and Nagai, 1973). The results obtained in the present study demonstrated that placing a skeletal muscle in Ringer's solution with an elevated calcium concentration immediately following the hypertonic urea incubation antagonized the block in e-c coupling. A preliminary report on some of these findings has been published (Frank and Treffers, 1977).

5. Electrophysiological Considerations

Many properties of skeletal muscle have been explained in terms of properties of the transverse tubular system; in particular the membrane capacitance (Falk and Fatt, 1964; Gage and Eisenberg, 1969a; Hodgkin and Nakajima, 1972b) the early after potential (EAP) following a single action potential (Gage and Eisenberg 1967, 1969b), the late after potential (LAP) following a train of action potentials (Freygang et al., 1964), and the slow potential change or 'creep' produced by a prolonged hyperpolarizing current (Adrian and Freygang,

1962). The observation that disruption of the T-system induced by glycerol-removal treatment abolished the EAP, LAP and creep (Gage and Eisenberg, 1967, 1969b) and greatly reduces the membrane capacity (Gage and Eisenberg, 1969a), strongly implies that these electrical parameters are associated with the transverse tubules.

Measurements of the effective membrane capacity ($\mu F/cm^2$ of surface area) in fast skeletal muscle fibres of the frog were higher (5 $\mu F/cm^2$, Katz, 1948; 8 $\mu F/cm^2$, Fatt and Katz, 1951) than measurements reported for slow striated muscle fibres (2.5 $\mu F/cm^2$, Adrian and Peachey, 1965) or for nerve (1 $\mu F/cm^2$, Curtis and Cole, 1938; Hodgkin and Rushton, 1946; Hodgkin et al., 1952). The relative high values in frog fast skeletal muscle fibres were consistent with the presence of a well developed T-system. On the other hand transverse tubules in frog slow skeletal muscle are sparse and nerve fibres do not posess a T-system at all. Falk and Fatt (1964) have separated the capacitance of frog sartorius fibres into two components by measuring the impedance of muscle fibres over a range of frequencies. They suggested that a large part of the capacitance resides in the tubular membrane (C_t: 4.1 $\mu F/cm^2$) and assigned the value of 2.6 $\mu F/cm^2$ to the surface membrane (C_s). Similar values for C_t (3.9 $\mu F/cm^2$) and C_s (2.2 $\mu F/cm^2$) were obtained by Gage and Eisenberg (1969a) who compared the electric properties of glycerol-removal treated fibres with those of normal fibres. Glycerol-removal treatment (Howell, 1969; Eisenberg and Eisenberg, 1968) functionally disconnects the T-system from the surface membrane and thus allows the separate capacities of the surface and transverse tubular membranes to be determined. Hodgkin and Nakajima (1972 a, b) investigated the passive electrical properties of the T-system

from a different approach. They studied the dependence of fibre diameter on low and high frequency capacity measurements and showed that the capacity measured with rectangular current pulses (low frequency capacity) increased with fibre diameter while estimates of the high frequency capacity (determined from the exponential rise of the foot of the action potential and conduction velocity) did not change according to the fibre diameter. Since the membrane area of the T-system increases in proportion with the diameter of the fibre, the effective capacity of a muscle cell expressed per unit area of surface membrane would be expected to increase likewise. It was concluded that the low frequency capacity represented the total capacity of the fibre (C_s+C_t) while the high frequency capacity measured the surface membrane capacity (C_s) only. All these observations point to the contribution that the T-system makes to the overall effective membrane capacity of a muscle fibre and indicate that transverse tubular and surface membranes are continuous.

Hodgkin and Horowicz (1960) showed that repolarization of the membrane potential of single fibres following a sudden decrease in potassium concentration followed a much slower time course than would be expected from the time constant of the muscle membrane. The slow time course of repolarization was consistent with the diffusion of K^+ ions from a space whose volume corresponded well with that of the T-system. In agreement with these findings (Nakajima et al., 1969, 1973) later showed that disruption of the T-system induced by glycerol-removal treatment also abolished the slow repolarization following the reduction of external $[K^+]$. Accumulation of potassium-ions in the T-system is also indicated by the work of Freygang et al. (1964).

These workers studied the longlasting depolarization (LAP) which occurs after muscle fibres are stimulated tetanically. The LAP is thought to be caused by an accumulation in the transverse tubules of potassium ions which leave the fibre during each successive action potential. Because of diffusion delays caused by the long and narrow tubular system, the potassium ions take some time to be diluted to the normal extracellular potassium concentration. Thus the observed slow decline of the LAP (T 1/2 = 350 msec) to normal resting potential levels can be explained in terms of the slow diffusion of K^+-ions to the exterior of the muscle fibre. Kirsch et al. (1977) showed that the slow decline of LAP (Freygang et al., 1964) and potassium repolarization (Nakajima et al., 1969, 1973) occurred at similar rates and confirmed that both phenomena could be explained by the potassium accumulation theory. In a similar fashion, the 'creep' which is a slow increase in potential to a more hyperpolarized value when a muscle is subjected to a prolonged inward current has been attributed to a progressive depletion of potassium ions from the tubular system (Adrian and Freygang, 1962). A further investigation of these slow conductance changes was carried out by Almers (1972 a, b) who showed that the 'creep' potential consisted of two components. The first component is noticable at membrane potentials less than -120 mV and is exclusively caused by depletion of K^+-ions within the T-system. At more negative potentials the decline in potassium conductance does not only arise from K^+-ion depletion but also from a voltage dependent decrease in potassium conductance. Barry and Adrian (1973) suggested that measurements of the magnitude of the 'creep' could be used as a diagnostic tool to monitor changes in tubular system parameters. In our studies

the creep and the LAP were selected as a means to assess the functional state of the transverse tubular system under conditions of urea- or glycerol-removal treatment.

CHAPTER II. MATERIALS AND METHODS

1. Tissue Preparation

Extensor longus digiti IV (toe) muscles and sartorius muscles of the frog, Rana pipiens, were used in the experiments. Each frog was decapitated, pithed and the toe and/or the sartorius muscles were removed from the frog. The toe muscle was dissected free from neighbouring muscles to which its tendons were attached. The connections of both ends of the toe muscle with surrounding muscles were left intact and were used to tie the toe muscle to a silk thread without damaging the muscle fibres. The toe muscle was then placed in a dissecting dish and further freed from connective tissue and other muscle tissue under a dissecting microscope. The sartorius muscle, dissected from the frog with a portion of the pelvic girdle was placed in a dissecting dish and some remaining connective tissue was carefully removed under the dissecting microscope. The inner surface of the sartorius muscle was used for microelectrode work since this side of the muscle contains less connective tissue than its superficial surface. Muscles were allowed to equilibrate for 30 minutes following dissection before an experiment was started. All experiments were carried out at room temperature (20° C).

2. Solutions

Ringer's solution with the following composition was used (in mM): NaCl, 111.8; KCl, 2.47; $CaCl_2$, 1.08; $NaH_2PO_4.2H_2O$, 0.44; $NaHCO_3$, 2.38; glucose, 11.1 and tubocurarine, 0.1 mg/ml. The osmolarity of this solution was 235 mOsm/kg (Osmometer, Advanced Instru-

ment Inc.).

High calcium (5 mM) Ringer's solutions were prepared by adding the appropriate amount of calcium chloride to the Ringer's solution. Hypertonic solutions of urea or glycerol were made which consisted of Ringer's solution in which 400 mM urea or glycerol was added to produce a total osmolarity of 635 mOsm/kg. The urea- or glycerol-removal treatment consisted of a one hour incubation in hypertonic urea or glycerol followed by reimmersion of the muscle preparation into an isotonic Ringer's solution.

3. Mechanical Recordings

For twitch recordings toe muscle preparations were mounted vertically in a 8 ml bath containing Ringer's solution. The lower end of the muscle was fixed near the bottom of the bath and the upper end was attached to the arm of a strain gauge by means of a silk thread.

Supramaximal, rectangular pulses, 2-5 milliseconds in duration were applied to the muscles by means of two platinum electrodes, one situated at the bottom of the bath and the other at the top of the bath. In most cases stimuli were applied every 30 seconds throughout the experiments.

The tension produced was recorded with a strain gauge whose active elements consisted of two pixie transducers in a wheatstone bridge configuration. The output was recorded with a Speed-servo recorder (Esterline Angus), a Beckman type R dynograph or an oscilloscope (Tektronix, type 565). Values of the resting tension on muscles ranged from 150-200 mg. Muscles were aerated with a mixture of 5%

CO_2 and 95% O_2.

4. Electrical Recordings

The two electrical parameters that were measured were the late after potential and the 'creep' potential. These were monitored with conventional glass micro-electrode techniques.

4.1 Electrodes. The glass micro-electrodes were drawn by a micro-pipette puller (Narishige, Japan) from melting point capillary tubes of 1.5-2 mm diameter. The settings of the micro-pipette puller were adjusted such that the tip length of the electrodes measured approximately 6 mm.

The glass electrodes were filled with a solution of 3 M KCl using a modified version of Tasaki's technique (1954). The electrodes were mounted on a microscope slide held together with a rubber band and were then put in a jar with their tips facing down. The jar was filled with methanol and the solution was boiled under reduced pressure by placing the jar in a vacuum desiccator for 10 minutes. Methanol was subsequently replaced by distilled water and the jar was left overnight at atmospheric pressure. The following day the jar was filled with 3 M KCl which had been filtered through a millipore filter with pore size of 0.45 μm. The electrodes were left in 3 M KCl for a minimum of 24 hours before use. The resistance of the micro-electrodes was measured (Danameter, model 2000, Dana Laboratories Inc.) and only those electrodes having resistances between 10 and 40 MΩ were selected for use.

4.2 Late After Potential (LAP). For measurements of the LAP, muscles were mounted horizontally in a bath and viewed from above with a dissecting microscope (Figure 4). Illumination was provided by light passing up through the glass bottom of the bath. In order to reduce muscle movement and consequent membrane damage during the recordings of the after potentials, toe muscles were stretched around a glass rod of 2 mm diameter, which was suspended across the bath in the Ringer's solution (Stefani and Schmidt, 1972).

Muscle fibres were stimulated electrically by means of two platinum wires (75 µm in diameter and 150 µm apart) which were insulated to their tips with a coating of teflon. Function generators (Tektronix 160 series and Grass SD9) were arranged to provide trains of 10 pulses. A stimulus isolation unit (Bioelectric Instruments Inc.) was used to reduce the stimulus artifact. The stimulating pulse consisted of a train of 10 individual square waves of 0.2 millisecond duration and 10 milliseconds apart. The stimulus strength was adjusted so that 10 successive action potentials were fired with each pulse train. Action potentials, resting membrane potentials and late after potentials were recorded by means of glass capillary micro-electrodes filled with 3 M KCl. The glass micro-electrode was held in a micro-electrode holder (W-P Instruments, model EH-1S) which was connected through a probe to the main amplifier (W-P Instruments, model M-701). The output from the amplifier was displayed on a type 565 dual beam Tektronix oscilloscope at two different sweep speeds (Figure 4). Only the fibre under study and sometimes a few nearby fibres were stimulated. The slow repolarization to resting membrane levels that follows the burst of action potentials, was monitored at

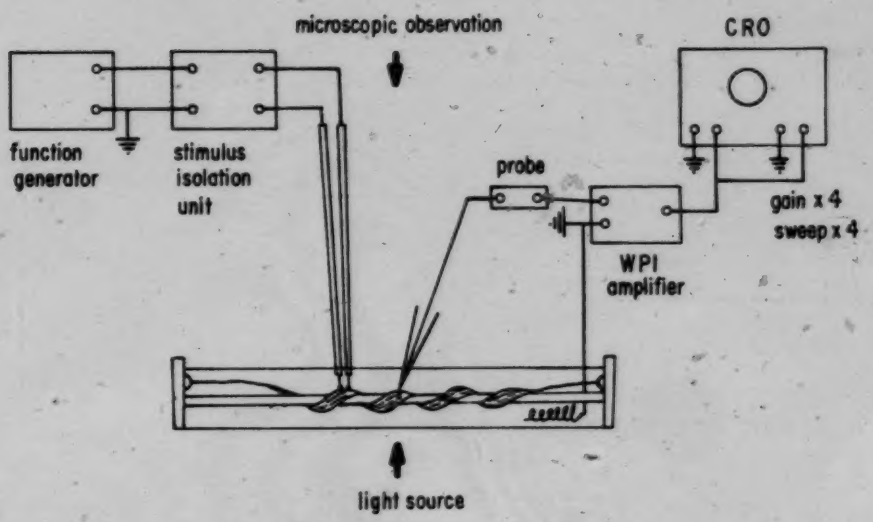


Figure 4. Experimental set up for the recording of Late after potentials in toe muscle fibres. Stimulating pulses were delivered to the muscle by means of extracellular Pt electrodes resting on the fibre surface. Action potentials and the late after potential were recorded intracellularly by means of a glass microelectrode. The electrical signal was amplified and displayed on the oscilloscope at different gains and sweepspeeds. The toe muscle preparation is wrapped around a glass bar several times to prevent excessive movement. CRO, cathode ray oscilloscope. See text for further details.

10 regular intervals over a time period of 888 milliseconds. The first measurement (time zero) was recorded 35 milliseconds after the last stimulating pulse. Measurements were made from a 35 mm film whose image was projected on a screen to provide a twelve-fold magnification. The above procedure was kept constant throughout the course of the experiments.

4.3 Creep Potential. Muscles were mounted horizontally in the tissue bath, supported by a glass pedestal at the site of electrode penetration (Figure 5). Two micro-electrodes were inserted into the same fibre approximately 50 μm apart. A rectangular hyperpolarizing current pulse of 2 seconds duration was passed through one electrode and the resulting displacement of membrane potential was measured with the other electrode. The current passing system was designed to check proper insertion of the micro-electrode into the cell and to monitor the current that was passed through the micro-electrode.

A constant current generator was used for passing current (Philbrick, 1966). The advantage of a constant current source is that the output current remains constant regardless of changes in micro-electrode resistance. This feature is extremely useful in situations in which long duration stimulating pulses must be delivered, since the micro-electrode changes its resistance during such prolonged pulses. An added advantage of the constant current generator is that higher resistance electrodes, which produce less membrane damage can be used. The arrangement of the stimulating and recording electrode assembly is shown in Figure 5 and the electrical circuitry of the constant current generator and the current monitor is shown in Figure 6.

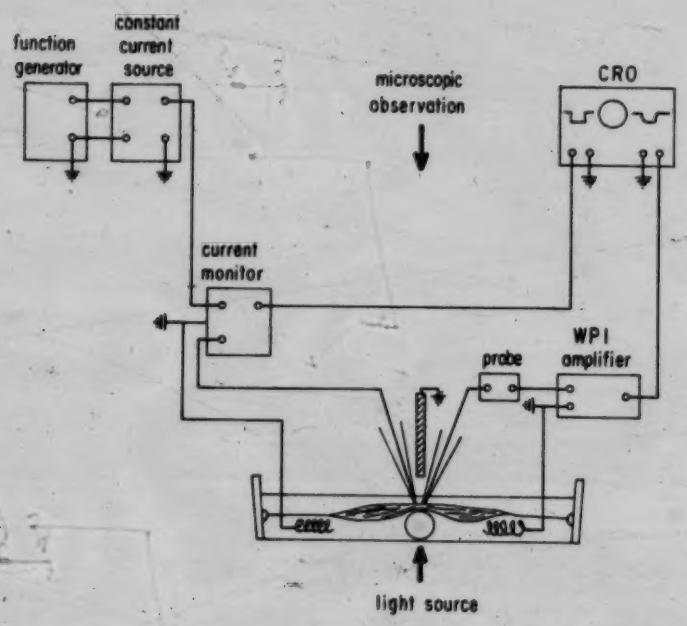


Figure 5. Arrangement of stimulating and recording electrode assembly designed to monitor creep potentials. Constant hyperpolarizing current pulses were passed intracellularly into the fibre through one electrode and subsequent membrane displacements were recorded through the other electrode placed in the same fibre. The current monitor fulfills a dual purpose: 1) it can be used to monitor the current passed through the stimulating electrode, 2) it can also be used to monitor resting membrane potentials (see Figure 6). CRO, cathode ray oscilloscope. See text for further details.

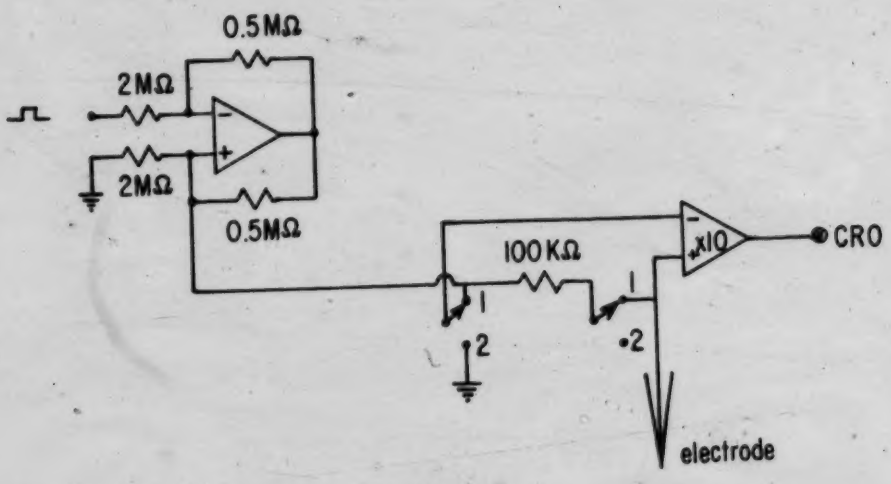


Figure 6. The electrical circuitry of the constant current generator and the current monitor. The operational amplifier in the upper left hand corner is connected as a constant current source. The operational amplifier in the right hand corner has a gain of 10 and is connected via a double pole, two position switch as a current monitor (position 1) or a membrane potential monitor (position 2) for checking proper electrode insertion. See text for further details.

The stimulating electrode was selected to have a resistance of approximately 10-20 MΩ and was connected to the input of the current monitor by means of a chlorided silver wire that contacted the 3 M KCl solution of the electrode. The electrode was held in place with a rubber stopper fitted in the shaft of the electrode holder (Figure 7), which in turn was attached to a micro-manipulator. Another micro-manipulator held the probe of the recording electrode.

Both manipulators were positioned such that the micro-electrode tips were close together, just above the muscle fibre membrane. The recording electrode was first inserted into the fibre and a sudden drop in resting membrane potential indicated a successful penetration. This was followed by the lowering of the stimulating electrode while the switch of the current monitor was in position 2 (Figure 6). Again the insertion of this electrode was monitored as a sharp drop in resting membrane potential. In order to check that both electrodes were situated in the same fibre the switch of the current monitor was changed to position 1 and a hyperpolarizing current was applied. The presence of an electrotonic potential detected by the recording electrode indicated that both stimulating and recording electrodes were in the same cell. The stimulating current was measured as a drop in voltage across a 100 KΩ resistor.

5. Electron Microscope Preparation

5.1 Conventional Electron Microscope Preparation. Toe muscles were used for all electron microscopic work and these muscles were fixed in 5% gluteraldehyde buffered at pH 7.4 with Millonig's

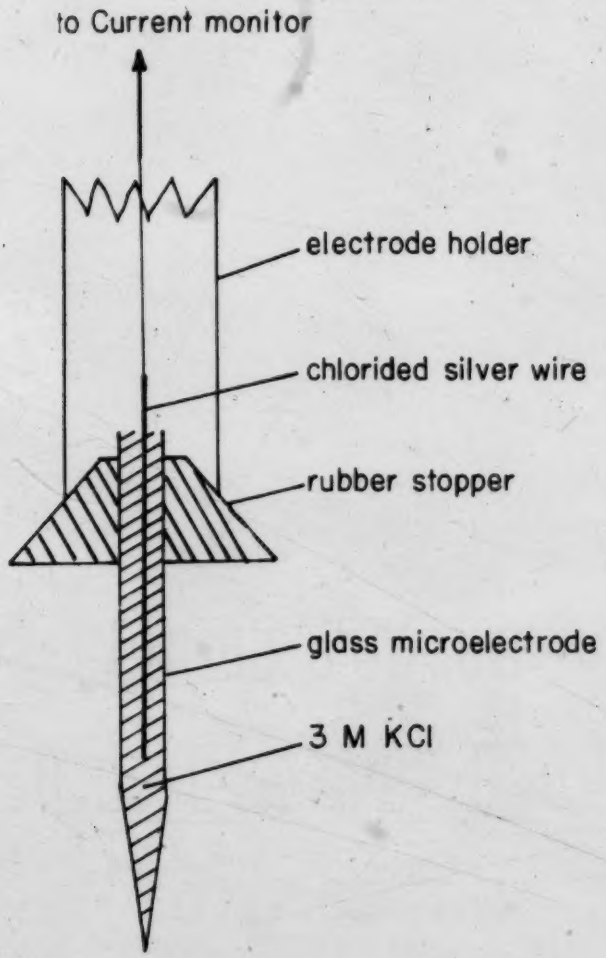


Figure 7. Stimulating electrode assembly. The glass microelectrode was held in place with a rubber stopper which fitted the electrode holder. The chlorided silverwire was connected to the current monitor. See text for further details.

phosphate buffer (Millonig, 1961). Thereafter, the muscles were removed from the bath and stored at 4° C in a buffered gluteraldehyde solution until muscles from several experiments had accumulated. Prior to postfixation the muscles were thoroughly washed in phosphate buffer and cut across into strips of tissue 3-4 mm in length. Tissues were postfixed for 40 minutes at 4° C in 1% osmium tetroxide buffered with Millonig's buffer solution. The tissue strips were rinsed in three 5 minute changes of distilled H_2O and were dehydrated in graded concentrations of ethanol followed by propylene oxide and were embedded in Epon resin.

The dehydration and embedding procedure was as follows: dehydration in successive solutions of 50%, 70%, 95% ethanol for 15 minutes in each concentration, followed by two 10 minute incubations in 98% ethanol; further dehydration in two 10 minute changes of propylene oxide and a 3 hour immersion in a 1:1 mixture of epon and propylene oxide. The mixture was replaced by epon and the tissue strips were left overnight. Finally the epon was allowed to set in molds and left to cure in an oven for two days at 60° C.

The epon resin consisting of: epon 812 epoxy resin, 15 ml; dodecenylsuccinic anhydride, 15 ml; nadic methylanhydride, 5 ml and DMP-30, 0.35 ml (Polysciences Inc.) was thoroughly mixed before the immersion of the tissues.

Longitudinal sections of the superficial fibres were cut on a Porter-Blum MT-2 ultramicrotome using glass or diamond knives. The sections had a thickness of approximately 60 nm as judged by their silver-grey color when floating in the collecting trough of the knife assembly. The sections were mounted on copper grids of 300-mesh, and

stained with uranyl acetate (15 minutes) followed by lead citrate (5 minutes) and were examined with an AEI Corinth 275 electron microscope or in some cases with a JEM-7A electron microscope.

5.2 Extracellular Markers. For comparison of the effects of a urea- or glycerol-removal treatment on the distribution of Ferritin/Horseradish peroxidase in the T-system, two toe muscles of the same frog were used in all cases. One muscle was always subjected to the particular urea- or glycerol-removal treatment while its untreated counterpart served as a control. Both muscles were subsequently exposed to the same extracellular marker and processed for electron-microscopy in an identical fashion.

i. Ferritin: The commercial solution of Ferritin (Horse spleen ferritin, twice recrystallized; Nutritional Biochemicals Corporation) was further purified by a method described by Huxley (1964). The purification procedure consisted of centrifugation of the commercial solution for 2 hours at 50,000 rpm (R_{max} = 218,010 g; R_{av} = 159,315 g) in a Beckman L2-65B ultracentrifuge. The resultant pellet was suspended in Ringer's solution and taken through 2 further cycles of sedimentation being redissolved each time in Ringer's solution. The final concentration of Ferritin was arranged to be approximately 20% w/v. For extracellular marker studies toe muscles were incubated in a Ferritin-Ringer's suspension for 30 minutes and subsequently prepared for electron-microscopy as described previously (section 5.1).

ii. Horseradish Peroxidase: Horseradish peroxidase type II (Sigma Chemical Co.) was the other marker molecule used in these

studies. The method for staining extracellular spaces is similar to that described by Eisenberg and Eisenberg (1968). Toe muscle preparations were incubated for 30 minutes in Ringer's solution to which 0.05% horseradish peroxidase had been added. In the case of urea- or glycerol-removal treated preparations, the muscle was first incubated in the hypertonic medium, then returned to isotonic Ringer's solution for 30 minutes and subsequently incubated in horseradish peroxidase Ringer's for 30 minutes. Following the 30 minute exposure to horseradish peroxidase, the muscle was fixed in cold phosphate buffered gluteraldehyde (3%) prepared from a 70% gluteraldehyde stock solution (LADD Research Industries) for 90 minutes. After gluteraldehyde fixation and three 5 minute buffer washes the muscles were placed in a reaction mixture containing: 10 ml (0.05 M) tris HCl buffer (pH 7.6); 5 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma and Polysciences Inc.) and 0.1 ml H_2O_2 (1%) prepared freshly from a 30% H_2O_2 stock solution. This mixture is stable for at least one hour and the incubation time was 30 minutes. During the exposure to the reaction mixture the muscle preparations were kept away from excessive light (Forssmann, 1969). Three 5 minute washes in distilled H_2O preceeded a 45 minute postfixation period at 4° C in 1% osmium tetroxide in phosphate buffer.

Subsequent dehydration and embedding were done as outlined in section 5.1 with the exception of uranyl acetate staining (Rubio and Sperelakis, 1972).

6. Measurements

6.1 Triad Width. The triad apparatus is oriented radially around the individual myofibrils and hence longitudinal sections of

the muscle will yield one of the following views of the triad:

a. When the triad is cut in cross section. The plane of section will be perpendicular to the long axis of the transverse tubule (Figure 26A).

b. When the triad is cut tangentially. The plane of section runs parallel to the long axis of the transverse tubule (Figure 26C).

c. Anywhere in between these two extremes (Figure 26B).

For measurements of triadic width micrographs were collected at random with any of the above views. Negatives were enlarged three times (x 180,000 magnification) and printed on photographic paper. Measurements were made of the distance between the outer surface of the T-tubule and the inner aspect of the membrane of the terminal cisternae (i.e. width of triadic junction + two times the width of the unit membrane) by means of vernier calipers. An estimate of the width of the triad junction was obtained by subtraction of 140 Ångstrom (two times the width of the unit membrane) from the above measurements. From each photograph of a triad junction four measurements were made (two from each junctional face of the triad) and the mean of these four measurements was recorded. Measurements were made only at positions where the opposing membranes were running parallel and a satisfactory resolution of the membranes was obtained. A total of ten micrographs of different triad structures was selected at random from each muscle and measured. The ten means were pooled to produce a mean which was considered to be representative of the population mean of the muscle in question.

6.2 Structural Integrity of the T-system. Estimates of structural integrity of the T-system in different toe muscles were obtained as follows. Muscles were prepared for electron-microscopy in the usual fashion (section 5.1) and longtitudinal sections were examined under the electron microscope. From each muscle six fibres were selected at random from different grids. The number of 'intact' transverse tubular sites were counted for every twenty myofibrils when the fibre was scanned along the Z-line from the sarcolemma inwards. The average value of 3 such scans for each fibre was entered in Table 4. Criteria for an intact transverse tubular site were: (i) The presence of one transverse tubule and at least one terminal cisterna; (ii) A clear resolution of the T-tubular and cisternal membranes at the junctional region.

CHAPTER III. RESULTS

1. Mechanical Observations

1.1 Urea-removal Treatment.

1.1.1 *The effect of a urea-removal treatment on the twitch response of frog's skeletal (toe) muscle.* The effect of a urea-removal treatment on the twitch is shown in Figure 8. A toe muscle was stimulated at constant intervals during the course of the experiment. Immediately upon immersion in the hypertonic urea solution there was a contracture response following which, after about 15 minutes, a small twitch reappeared reaching maximum tension approximately 60-70 minutes after immersion in the urea-Ringer solution. When the muscle was returned to a urea-free solution the twitch was initially potentiated but disappeared within 20-25 minutes of urea-removal. Despite the loss of twitch urea-removal treated muscles were still able to respond mechanically to caffeine although the maximum tension was reduced slightly (Figure 9). Urea-removal treatment did not always abolish the twitch response. Experimental data collected over a two-year period showed that out of 27 toe muscles subjected to a urea-removal treatment with 400 mM urea only 11 muscles exhibited a complete loss of the twitch. The non-uniformity of the mechanical response of frog muscles to a urea-removal treatment could not be attributed to seasonal variation but instead may have been related to muscle size. However this possibility remains to be investigated since no measurements of muscle diameter were performed in this thesis. When the concentration of urea was increased to 600 mM, the twitch response was abolished in 6 out of 8 toe muscles and also the

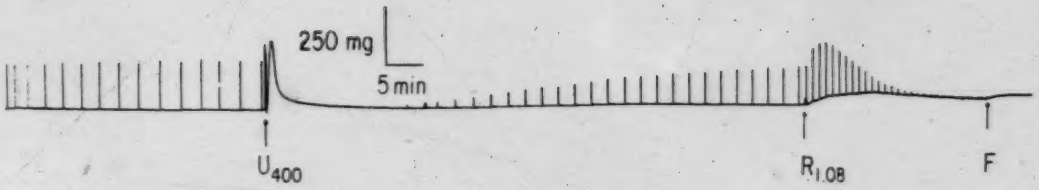


Figure 8. Effects of a urea-removal treatment on the twitch response of a frog's skeletal (toe) muscle. at U_{400} the muscle was immersed in Ringer's with 400 mM urea; at $R_{1.08}$ the muscle was placed in a urea-free Ringer's solution with normal calcium concentration (1.08 mM Ca). Muscles were stimulated once every 2 minutes preceeding and during the urea incubation. Following the urea-washout the stimulating frequency was increased two-fold. At F the muscle was placed in fixative solution and further processed for electron microscopy (see methods).

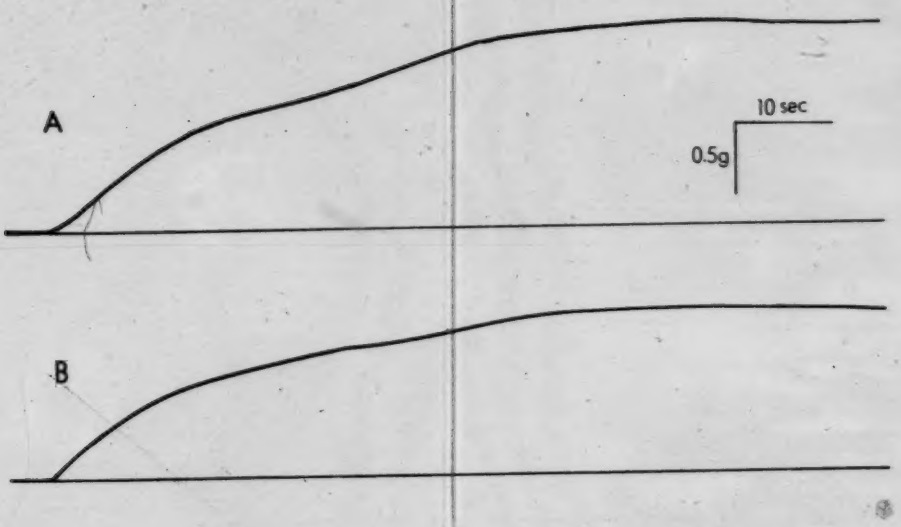


Figure 9. Caffeine (8×10^{-4} g/ml) contractures in a toe muscle before (A) and after (B) urea-removal treatment. Caffeine was still able to elicit a contracture at a time when the twitch response had completely disappeared (B).

rate of twitch decline was faster (Figure 10). When urea-removal treated muscles were left in Ringer's solution for a prolonged period of time the twitch slowly recovered to approximately 18% of pre-urea control values. In Table 1 partial recovery of twitch was observed for 6 muscles following a one hour exposure to a 400 mM urea-Ringer solution. No recovery of twitch tension took place when the urea concentration was increased to 600 mM.

It was found that urea-exposures as short as 15 minutes were sufficient to cause a reduction in twitch tension on return to Ringer's solution. The longer the time of exposure to urea the more rapid was the fall in twitch on return to Ringer's solution. The relationship between the 'load-time' in urea-Ringer and the rate of twitch reduction upon return to Ringer solution was recorded for a number of muscles. In Figure 11 the time for the decline of twitch tension to 25% of the pre-treatment control level is plotted against various load-times.

1.1.2 *Calcium antagonism of the loss of twitch following urea-removal treatment.* The loss of twitch was prevented when toe muscles immersed in hypertonic urea for 60 minutes were placed in a 5 mM calcium-Ringer solution. The antagonizing effect of the high (5 mM) calcium-Ringer solution on the loss of twitch tension is demonstrated in Figure 12 for a single experiment conducted on two toe muscles from a single frog. Figure 13 summarizes the results obtained from 10 such experiments and illustrates the average time course of decline of twitch tension under conditions of high and normal calcium concentrations. In contrast to its effect on urea-removal treated muscles, a

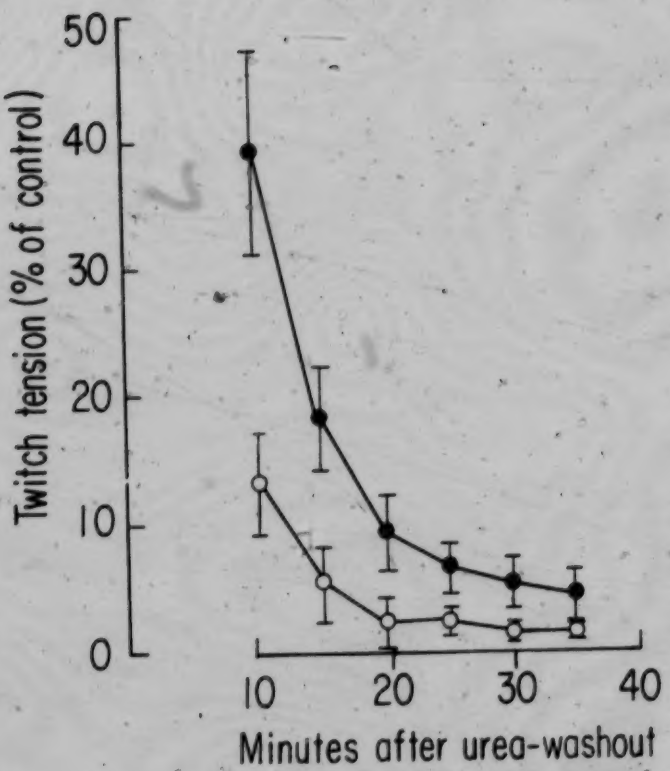


Figure 10. The effect of urea concentration on the decline of the twitch response in toe muscle. 5 muscles were used to calculate the mean ± SE for each point (see Table 1). •, muscles preexposed to 400 mM urea for 1 hr; o, muscles preexposed to 600 mM urea for 1 hr. The average decline of twitch tension on return to Ringer's solution is shown for the different preexposure concentrations of urea.

Table 1. Partial recovery of twitch tension following urea-removal treatment. Toe muscles were exposed to 400 mM urea for 1 hour and reimmersed in a normal Ringer's solution for 3-4 hours. Twitches were recorded at minimum tensions and 3-4 hours after return to Ringer's solution.

	Minimal twitch		Maximum twitch	
Muscle	Minutes	% Control	Hours	% Control
7/1	40-50	3	3-4	17
18/3	35-40	10	3-4	26
5/6	40-55	12	3-4	34
13/7	48	1	3-4	15
8/7	42	3	3-4	8
13/7	34	1	3-4	15
Average minimal twitch	34-55	5	3-4	18

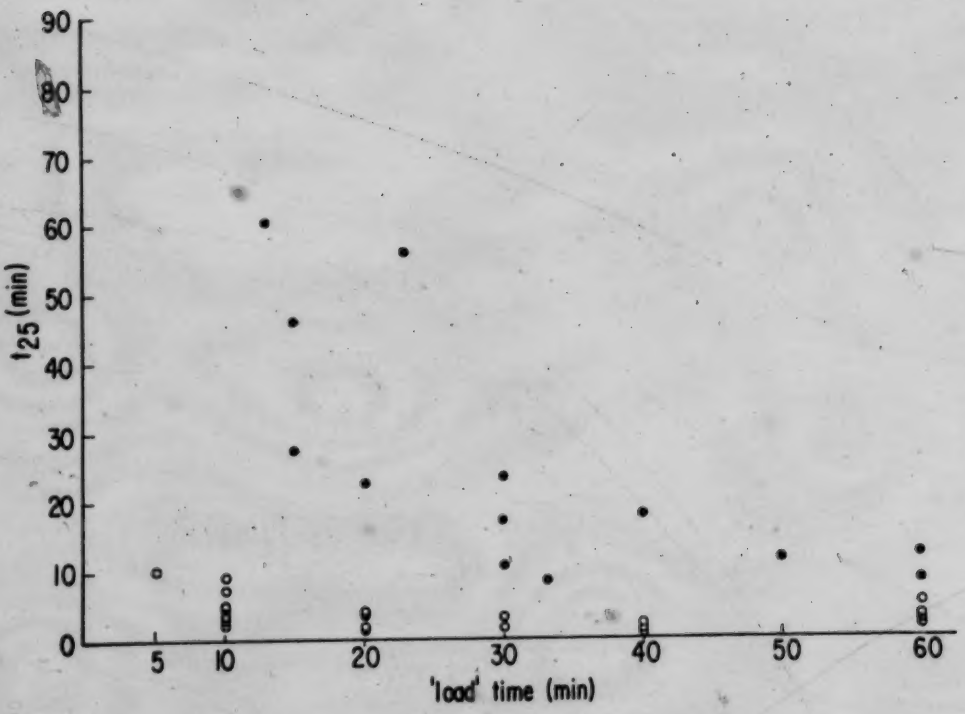


Figure 11. The relationship between the duration of exposure (i.e. load time) of toe muscles to hypertonic urea (•) or glycerol (o) solutions (400 mM) and the time at which twitch tension fell to 25% of the pre-treatment twitch tension (t_{25}), after the return to Ringer's solution.

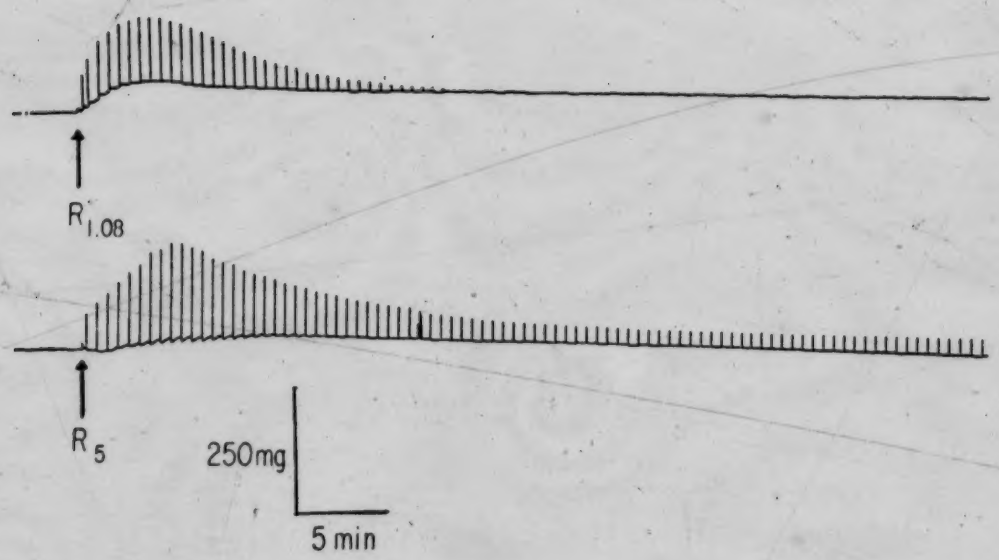


Figure 12. Prevention of the urea-removal treatment loss of twitch tension by elevation of the Ca^{++} concentration. Two toe muscles from the same frog were mounted in seperate baths and tested simultaneously. At $R_{1.08}$ (upper record) the muscle was placed in Ringer's with 1.08 mM Ca^{++} following a 60 minute exposure to 400 mM urea. At R_5 (lower record) the muscle was placed in Ringer's with 5 mM Ca^{++} following urea treatment. In all other paired experiments, the muscles were fixed within one minute after the twitch had disappeared in the muscle placed in Ringer's with 1.08 mM Ca^{++}.

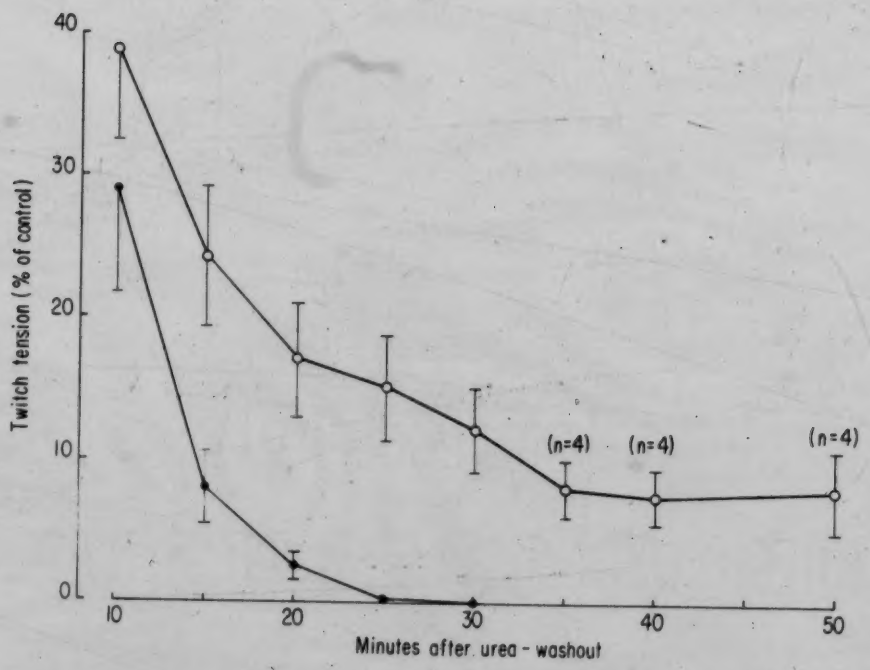


Figure 13. Time course of the decline in twitch tension in toe muscles following urea-removal. Except as noted, 10 muscles (n=10) were used to calculate the mean ± SE for each point. •, muscles placed in 1.08 mM Ca^{++} Ringer's; o, muscles placed in 5 mM Ca^{++} Ringer's. Control, twitch tension recorded for each muscle before a 60 minute exposure to the urea (400 mM) Ringer's solution.

five fold increase in calcium concentration in the solution bathing untreated muscles reduced the twitch by 25% (Figure 14). As mentioned above, when urea-removal treated muscles were left in Ringer's solutions for prolonged periods a partial recovery of twitch tension could be observed (Table 1). In Figure 15 partial recovery of twitch tension in urea-removal treated muscle was recorded under conditions of high and normal calcium. Recovery of twitch tension was enhanced under conditions of high calcium. When muscles were exposed to urea concentrations of 600 mM for one hour and were subsequently returned to a urea-free, high calcium-Ringer solution, the average decline in twitch tension was not significaltly different from muscles placed in a normal calcium-Ringer solution (Figure 16).

1.1.3 *The effects of a phosphate buffer substitution and the effects of cobalt ions on the twitch response of urea-removal treated toe muscles.* Frank (1978b) recently demonstrated that the twitch can be abolished rapidly when muscle fibres are placed in a calcium-free solution. He also showed that the twitch loss could be prevented by either the substitution of a phosphate buffer for a bicarbonate buffer or by the addition of cobalt ions (Frank, 1978a) to the calcium-free Ringer's solution. We have used these two experimental procedures to determine their effect on the urea-removal treatment-induced loss of twitch response. Two toe muscles from the same frog were exposed to urea (400 mM) for one hour. When urea was replaced with a urea-free Ringer's solution buffered with either phosphate (Figure 17 B) or bicarbonated (Figure 17 A) no difference in the loss of twitch could be detected between these two muscles. Thus it seemed that substitu-

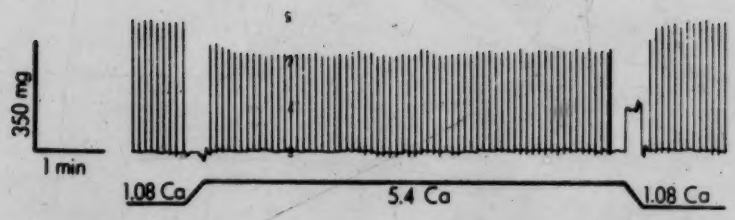


Figure 14. The effect of five-fold increase in Ca^{++} concentration on the twitch response in untreated toe muscle. The twitch tension was reduced by 25% after immersion in the high calcium concentration.

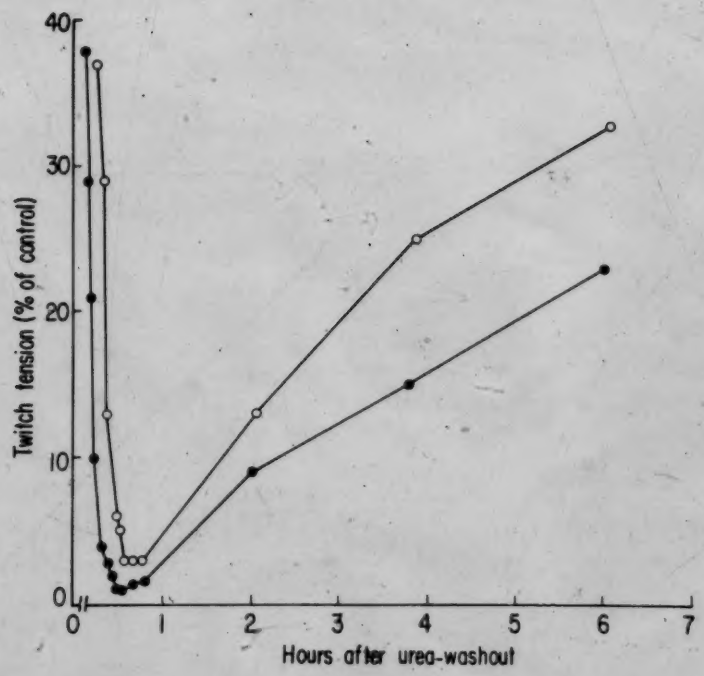


Figure 15. Partial recovery of twitch tension after urea-removal treatment under conditions of high and normal calcium. Two toe muscles from the same frog were exposed to a 400 mM urea-Ringer's solution for one hour after which they were returned to a high (5 mM) calcium-Ringer (o) or a normal (1.08 mM) calcium-Ringer (•) solution. Twitch tensions were recorded for a period of 6-7 hours following reimmersion in the urea-free Ringer's solution.

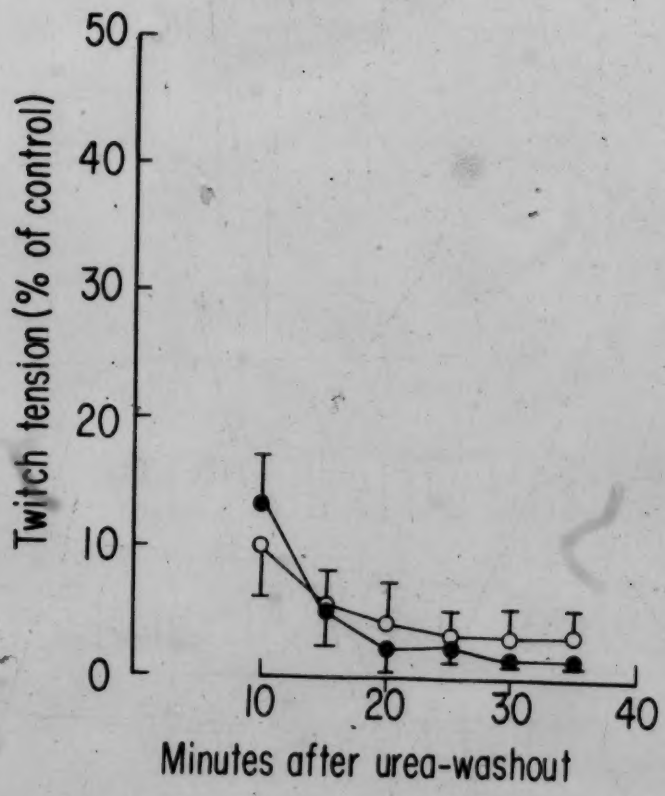


Figure 16. Time course of decline in twitch tension in toe muscles following urea (600 mM)-removal. Five muscles were used to calculate the mean ± SE for each point. •, muscles placed in 1.08 mM Ca^{++} Ringer's; o, muscles placed in 5 mM Ca^{++} Ringer's solution. Control, twitch tension recorded for each muscle before exposure to the urea Ringer's solution. No significant difference between the means was detected ($p > 0.05$).

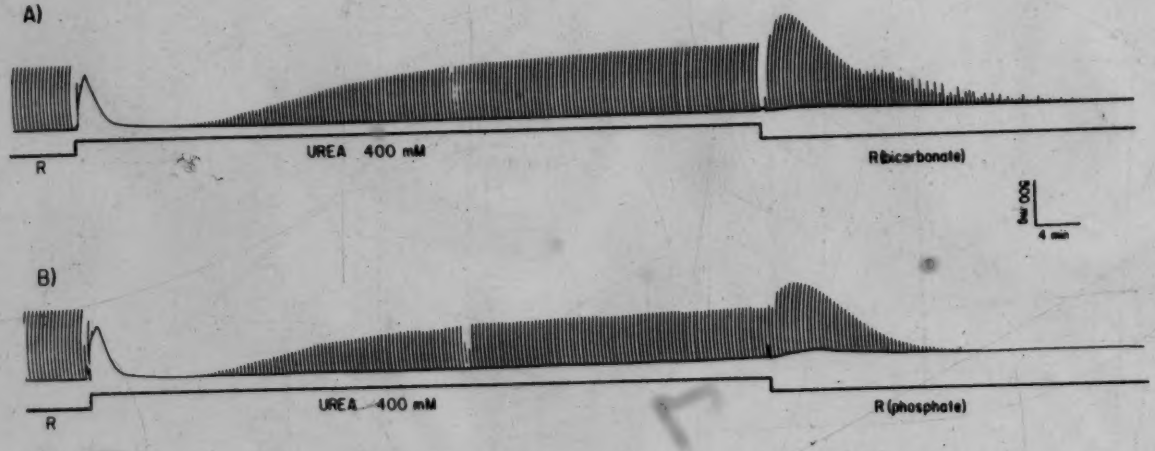


Figure 17. The effects of a phosphate-buffer substitution on the loss of twitch in urea-removal treated toe muscles. A, urea-removal treatment with the conventional bicarbonate-buffered Ringer's solution; B, urea-removal treatment in which the bicarbonate buffer was replaced with a phosphate buffer during the urea-washout. A and B were obtained from different muscles of the same frog.

tion of the phosphate buffer for the bicarbonate buffer did not antagonize the loss of twitch induced by a urea-removal treatment. Likewise, it was found that the addition of cobalt-ions during the urea-washout did not prevent the loss of twitch. Figure 18 A and B shows a typical experiment in which a comparison was made between the effects of a urea-removal treatment in two paired muscles (same frog), subjected to respectively a cobalt (5 mM) and a cobalt-free Ringer's solution. Other concentrations of cobalt, i.e. 0.1 and 1.0 mM, were also employed but were not able to prevent the loss of twitch response induced by a urea-removal treatment.

1.1.4 *The effect of a urea-removal treatment on the twitch response of frog's sartorius muscle.* Frog sartorius muscles were treated with a hypertonic urea-Ringer's solution for 1 hour followed by reimmersion of the muscle into a urea-free Ringer's solution containing either high (5 mM) or normal calcium concentration. In contrast to the effects of high calcium in the toe muscle preparation, the high calcium ringer's bathing the sartorius muscle did not significantly alter the reduction in twitch tension following the urea-washout (Figure 19). The average decline of twitch tension (n = 5) after urea-removal treatment was slower for sartorius than that for toe muscle.

1.2 Glycerol-removal treatment.

1.2.1 *The effect of a glycerol-removal treatment on the twitch response of frog's skeletal (toe) muscle.* Figure 20A shows the effects of a glycerol-removal treatment on the twitch response

Figure 18. The effects of cobalt-ions on the loss of twitch in urea-removal treated toe muscles. Urea-removal treatment in the absence (A) and presence (B) of cobalt (5 mM) in different muscles from the same frog.

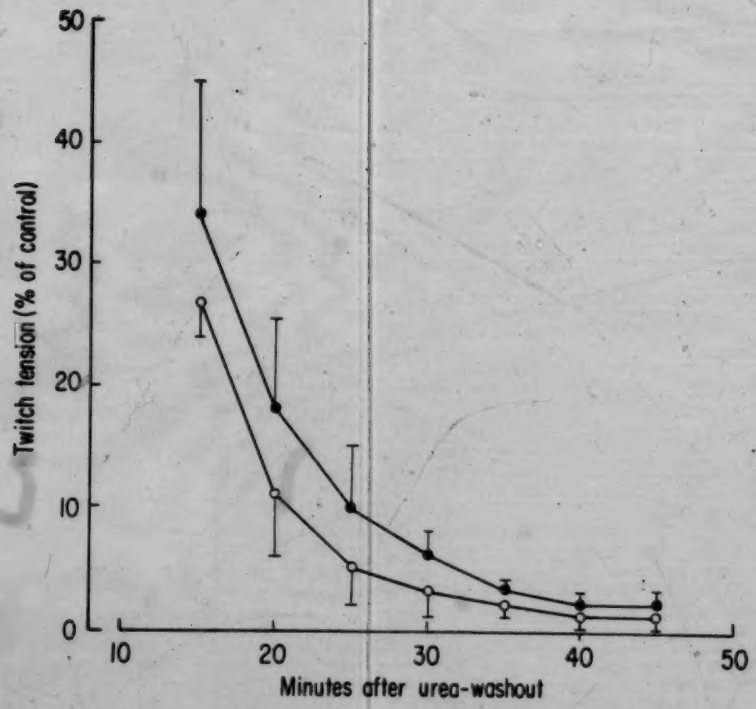


Figure 19. Time course of decline in twitch tension in sartorius muscles following urea-removal. Five muscles were used to calculate the mean ± SE for each point. •, muscles placed in 1.08 mM Ca^{++} Ringer's; o, muscles placed in 5 mM Ca^{++} Ringer's. Control, twitch tension recorded for each muscle before exposure to the urea (400 mM) Ringer's solution. No significant difference between the means was detected ($p > 0.05$).

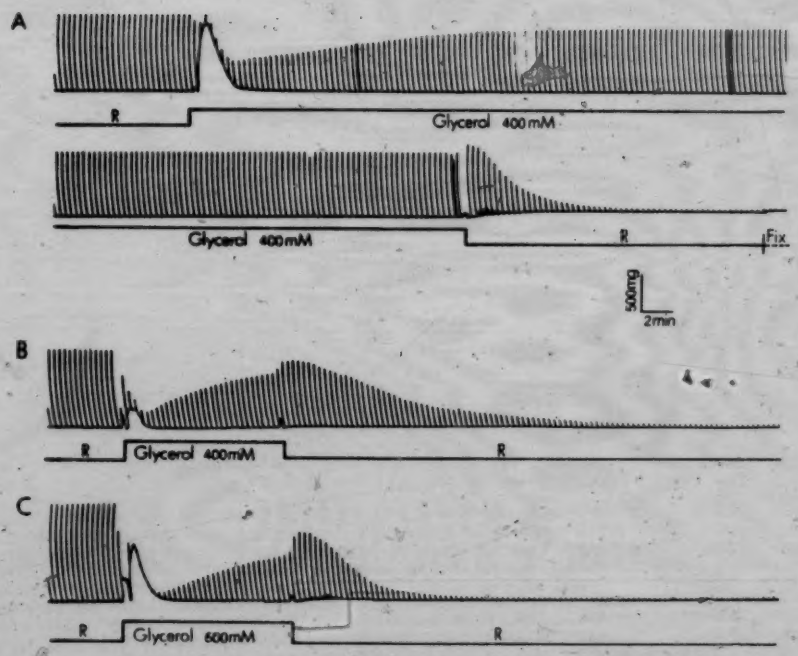


Figure 20. The effect of different glycerol-removal treatments on the twitch tension of frog's skeletal (toe) muscle. Muscles were stimulated at a rate of 3 pulses per minute throughout the experiment. A, long (60 min) glycerol (400 mM)-removal treatment; B, short (10 min) glycerol (400 mM)-removal treatment; C, short (10 min) glycerol (600 mM)-removal treatment.
Note that for all glycerol-removal treatments the twitch tension was greatly reduced but not completely abolished.

in toe muscle. On immersion of the muscle in the hypertonic glycerol-Ringer solution, a transient contracture with a simultaneous reduction in twitch tension was observed. Twitch tension recovered to 80% of pre-glycerol tension within 20 minutes. After one hour the hypertonic glycerol-Ringer's solution was replaced with a glycerol-free Ringer's solution and the twitch response was reduced to 10% of pre-glycerol tension in less than 15 minutes. In contrast to other reports in the literature (Howell, 1969; Dulhunty and Gage, 1973a) we never observed a complete loss of twitch tension following glycerol-removal treatment. It was found that the twitch was reduced to the same extent with a short (10 minute) glycerol-removal treatment (Figure 20B) and that increasing the glycerol concentration to 600 mM still did not abolish the twitch response (Figure 20C).

Unlike similar experiments with urea, no correlation could be shown to exist between the duration of the glycerol exposure and the time for the decline of twitch tension to 25% of the pretreatment control tension (Figure 11).

Despite the drastic reduction in twitch tension induced by a glycerol-removal treatment, the caffeine-induced contracture was only slightly diminished. Figure 21 shows the mechanical response to caffeine before (A) and after (B) glycerol-removal treatment.

1.2.2 *The effects of elevating the extracellular Ca^{++} concentration on the twitch response in glycerol-removal treated toe muscles.* It has generally been assumed (Howell, 1969; Eisenberg and Eisenberg, 1968) that glycerol-removal treatment is responsible for the swelling and subsequent sealing of the T-tubules from the extra-

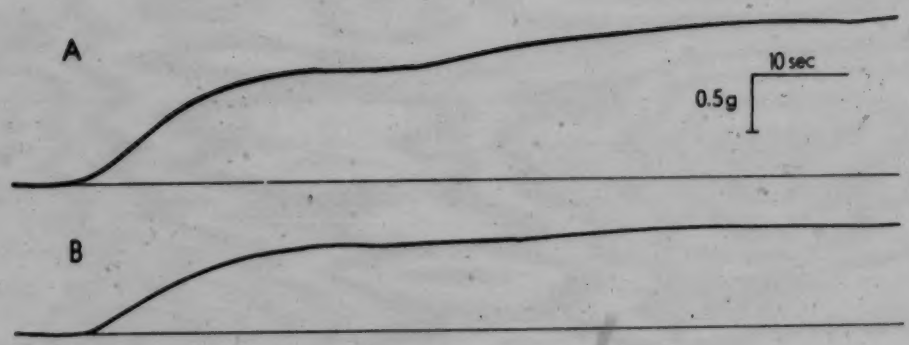


Figure 21. Caffeine (8×10^{-4} g/ml) contractures in a toe muscle before (A) and after (B) urea-removal treatment. The twitch response in B was reduced to less than 10% of the pre-glycerol twitch tension while a near maximum (70%) caffeine-contracture could still be elicited.

cellular space. It is the closure of the T-system which causes the inability of the muscle to contract in response to depolarization of the surface membrane. Dulhunty and Gage (1973a) presented evidence which indicated that the failure to twitch following a short glycerol-removal treatment occurred independently of a closure of the T-system. We have examined if the twitch failure resulting from short glycerol-removal treatments was of the same nature as the twitch loss observed for urea-removal treated muscles. In contrast to urea-removal treatment, the reduction in twitch tension of glycerol-removal treated muscles was not antagonized by a five-fold increase in Ca^{++} concentration following the glycerol-washout. This is illustrated in Figures 22 A, B, and C, which represent the average decline of twitch tension of muscles exposed to hypertonic glycerol for respectively 10, 20 or 40 minutes. No significant difference was detected between the decline of twitch tension of muscles reimmersed either in high (5.4 mM) or normal calcium. The average decline of twitch tension of muscles exposed to a 60 minute glycerol-removal treatment is shown in Figure 22D and is similar to the shorter duration glycerol-removal treatments (Figure 22 A, B, and C).

1.2.3 *The effect of glycerol-removal treatments on the twitch response of frog's sartorius muscle.* Frog sartorius muscles were immersed in hypertonic (400 mM) glycerol-Ringer's for 15, 30 or 60 minutes. Following the glycerol-exposure these muscles were placed in a glycerol-free Ringer solution containing either a high or a normal calcium concentration. Figures 23 A, B, and C show the average decline of twitch tensions under these conditions. In con-

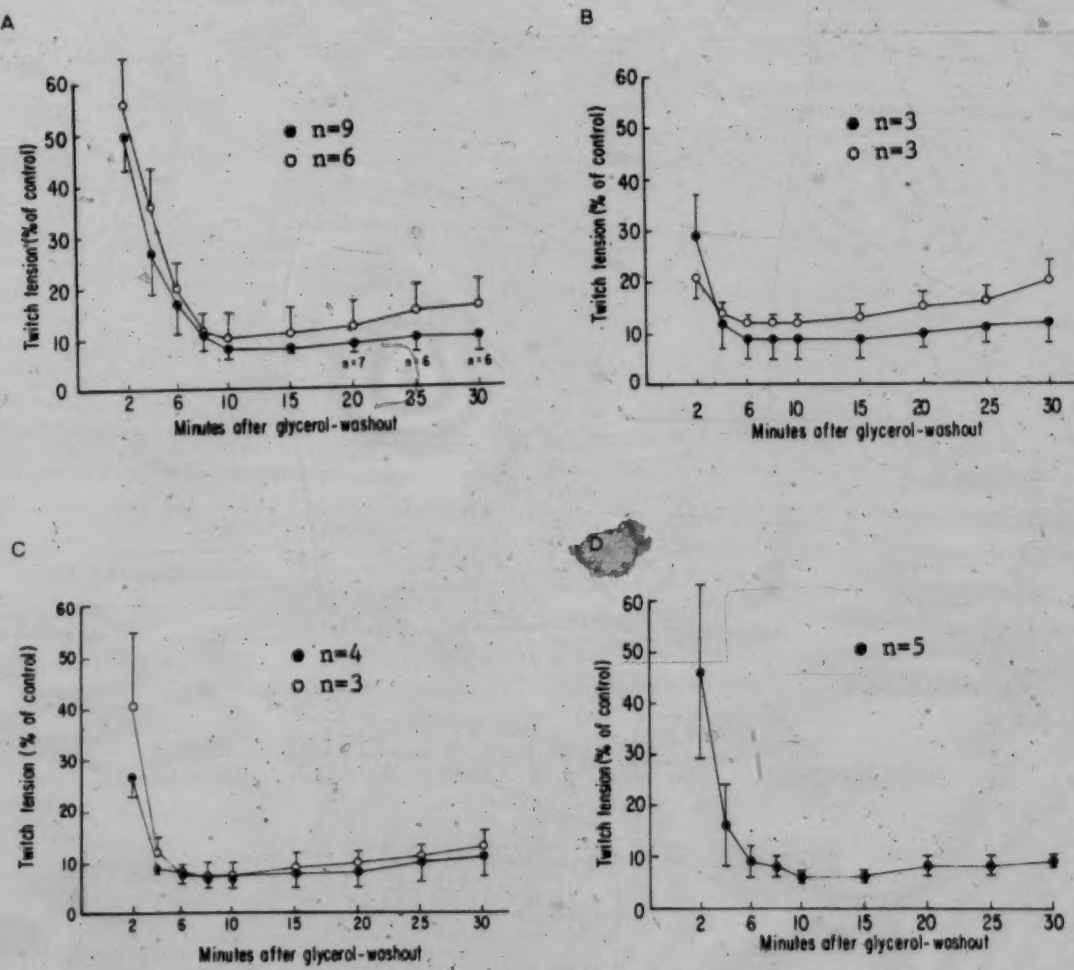


Figure 22. Time course of decline in twitch tension in toe muscles following glycerol-removal treatments of different duration. Muscles were exposed to glycerol (400 mM) and were subsequently placed in a glycerol-free Ringer's solution containing 1.08 mM Ca^{++} (•) or 5.4 mM Ca^{++} (o). The number of muscles (n) used to calculate the mean ± SE for each point is indicated in each diagram. A, B, C and D; respectively 10, 20, 40 and 60 minute glycerol-removal treatments. No significant difference between the means was detected ($p > 0.05$).

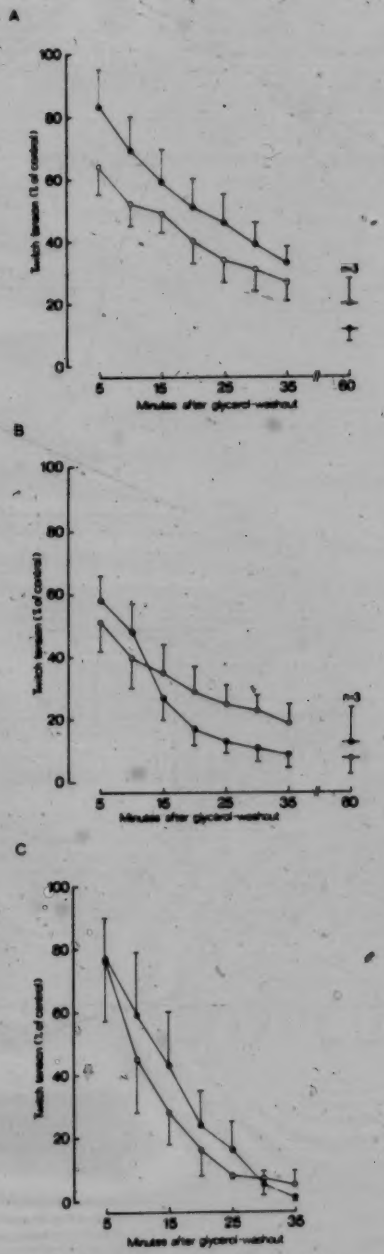


Figure 23. Time course of decline in twitch tension in sartorius muscles following glycerol-removal treatments of different duration. Muscles were exposed to glycerol (400 mM) and were subsequently placed in a glycerol-free Ringer's solution containing 1.08 mM Ca^{++} (•) or 5.4 mM Ca^{++} (o). Except as noted five muscles were used to calculate the means ± SE for each point. A, B and C; respectively 15, 30 and 60 minute glycerol-removal treatments. No significant difference between the means was detected ($p > 0.05$).

trast to a similar treatment following urea-removal (Figures 12 & 13), a five-fold increase in extracellular [Ca^{++}] did not antagonize the reduction in twitch tension following the glycerol washout. The significance of these results will be discussed later.

2. Electron Microscopic Observations

2.1 General Ultrastructure. Freshly excised toe muscles from the frog were fixed in 5% gluteraldehyde in phosphate buffer and processed for electron microscopy as described in the methods section. Silver-grey sections of tissue, where the fibres were cut in longtudinal orientation were examined under the electron microscope. Figure 24 represents a low magnification view of a muscle fibre showing the parallel stacking arrangement of the myofibrils. At regular intervals along the Z-lines, fragments of the T-system with two adjoining vacuoles can be seen. These 3 structures, transverse tubule and two terminal cisternae represent a triad and are shown at higher magnification in Figure 25. The Figures 26 A, B, and C show the different appearances of triads when muscles are sectioned longitudinally (see methods). The area interspersed between the membrane of the T-tubule and that of the terminal cisterna is the triad junction (Figure 26B).

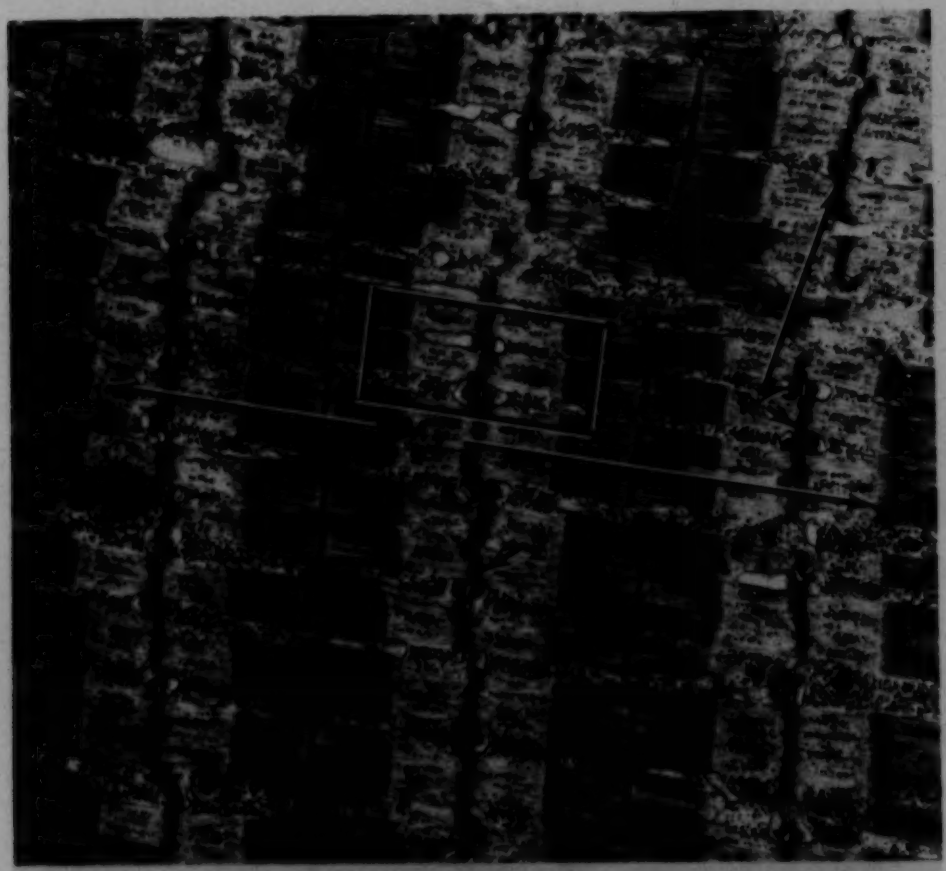
2.2 Urea-removal Treatment.

2.2.1 *Measurements of the triad junction width of urea-removal treated toe muscles.* From each micrograph of a triad structure four measurements of its junctional width were made and the mean

Figure 24. Longitudinal section of muscle fibre, showing myofibrils (My) and triads (Tr) located in the vicinity of the Z-lines. The area within the square is displayed in Figure 25 at a higher magnification.
Staining: Uranyl acetate (15 min), followed by lead citrate (7 min). X 18,000

Figure 25. High magnification view of the triad apparatus. Triads, consisting of two terminal cisternae (Tc) and one transverse tubule (Tt) are shown. The transverse tubules are superimposed on the Z-line (Z). The dark granules are glycogen granules (Gly). Longitudinal tubules (Lt) of the sarcoplasmic reticulum can also be seen. X 75,000

24



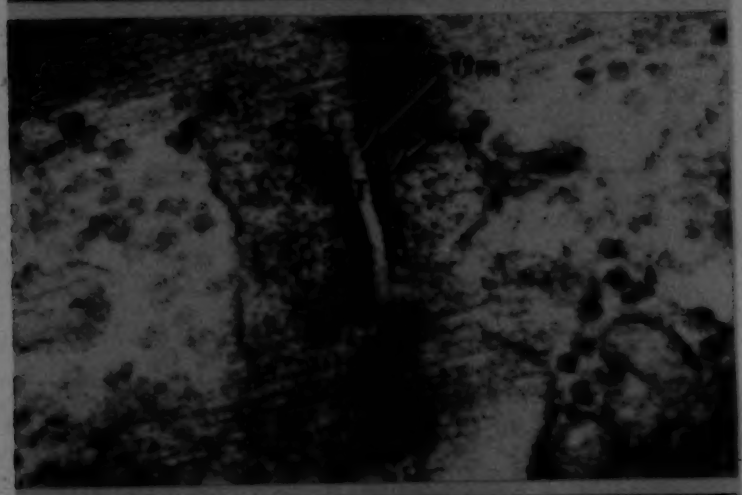
25

Figure 26 A, B, and C

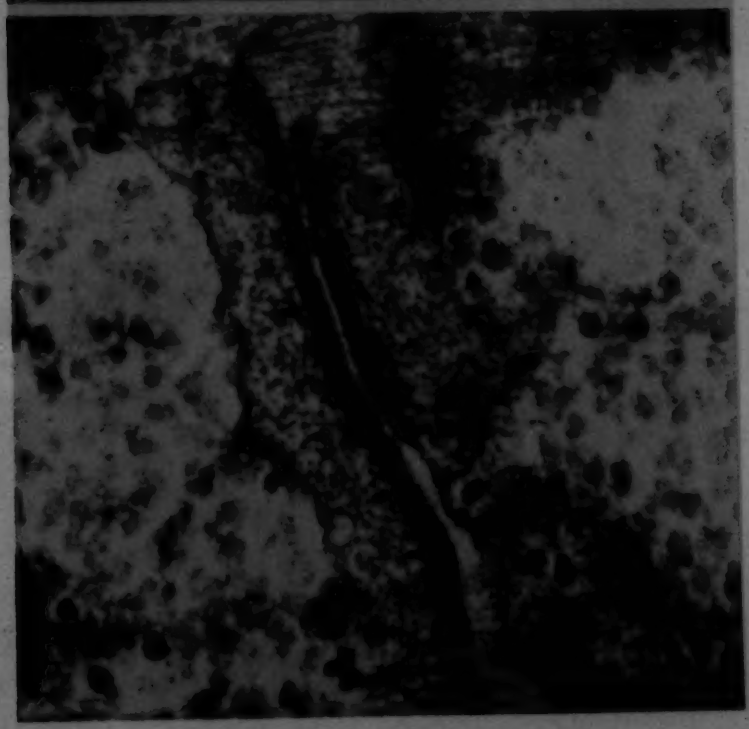
Different orientations of the triad apparatus. When a muscle fibre is sectioned longitudinally the following views of the triad may occur. A, is a triad sectioned perpendicular to the long axis of its transverse tubule (T). C, is a triad sectioned parallel to the long axis of its transverse tubule. B, is an orientation in between that of A and C. Note the triad junction, which is the region between the transverse tubular membrane (Ttm) and the membrane of the terminal cisterna (Tcm). Staining: Uranyl acetate (15 min) followed by lead citrate (5 min). X 180,000

26 A

B



C



of these four measurements was recorded. A total of ten micrographs of different triad structures was selected at random from each muscle and their triadic junctional widths were measured. An estimate of the mean triadic width for a single muscle (n = 10 x 4) was thus obtained (see methods). Toe muscles from the right and left foot of each frog were randomly assigned muscle 1 or muscle 2 for each pair. No significant difference was detected between triadic width measurements for the two muscles under the untreated condition (Table 2A). In the urea-removal treatment experiments both muscles in each pair were fixed simultaneously with gluteraldehyde buffer within a few seconds after the twitch response in muscle 1 (1.08 mM Ca^{++}) had disappeared. The twitch tensions recorded at this time for each muscle 2 (5 mM Ca^{++}) are listed in Table 2B. Despite the difference in twitch response produced by altering the Ca^{++} concentration (section 1.1.2, Figure 13) there was no significant difference between the widths of the triad junction in muscles 1 and 2. However, in confirmation of the findings of Oota and Nagai (1973), a significant increase in the width of the triad junction was produced by the urea-removal treatment.

2.2.2 *Distribution of Ferritin in urea-removal treated frog's skeletal muscle.* Urea-removal treated toe muscles were soaked in a Ferritin-Ringer's solution (20% w/v) for 30 minutes following a 30 minute urea-washout period. After the Ferritin-soaking period muscles were fixed in a 5% gluteraldehyde-phosphate buffer together with an untreated muscle from the same frog. The untreated muscles, also soaked in Ferritin served as controls. Both muscles were processed simultaneously for electron microscopy. Longitudinal sections

Table 2. Measurements of the width of the triadic junction in untreated toe muscles and in muscles subjected to the urea-removal procedure. Triadic junction width listed for each muscle is the mean ± standard error of the mean (SE) of 10 measurements (see Methods). Twitch tensions for urea treated muscle 2 recorded just prior to fixation when twitch tension in muscle 1 was zero.

A. Untreated toe muscles			B. Urea-removal treated toe muscles			
	Triadic width (Å)			Triadic width (Å)		Twitch tension
Expt. No.	Muscle 1	Muscle 2	Expt. No.	Muscle 1 (1.08 mM Ca)	Muscle 2 (5 mM Ca)	Muscle 2 (% of control)
4/12	91±5	84±4	17/9	119±4	122±3	16
5/12a	92±5	93±5	18/9	104±5	107±5	9
5/12b	91±3	93±5	22/9	116±5	102±4	2
6/12	80±4	89±5	23/9	121±5	98±3	7
			11/12	101±6	110±3	33
			23/12	114±3	102±3	18
			15/1	107±3	128±6	13
			16/1	114±3	109±3	23
Muscle means ±SE*	88.5±2.8	89.8±2.1		112.0±2.5	109.8±3.7	15.1±3.5
Treatment means ± SE**	89.1±1.7			110.9±2.2		

* Triadic width means for muscles 1 and 2 did not differ significantly in A or B: $P > 0.05$.

** Triadic width means for urea-removal treated muscles was significantly greater than for untreated muscles: $P < 0.001$.

were examined and marked differences in the distribution of the Ferritin were observed. Figure 27 is a low-magnification view of a urea-removal treated muscle in which the T-system is intact, although some vacuolization of the transverse tubules occurs. The extracellular space (ecs) is filled with electron-dense Ferritin particles while no Ferritin is visible in the T-system (Figures 28 and 29). In untreated toe muscles the accumulation of Ferritin particles in the transverse tubular system is clearly visible (Figures 31 and 32). Apart from occasional vacuolization of segments of the T-system (Figures 27 and 30) no differences could be detected between the gross ultrastructure of either urea-removal treated muscles or their controls. The dense material in the vacuolated T-system consists of glycogen granules (Figures 27 and 30) which have leached out from the sarcoplasm into the swollen segments of the T-system as a result of local membrane rupture. The dark spots on the glycogen granules (Figure 30) are distinct from Ferritin particles (Figure 32) and presumably represent a staining artifact since similar appearances were encountered in muscles not treated with Ferritin.

2.2.3 *Distribution of Horseradish peroxidase in urea-removal treated frog's skeletal muscle.* In contrast to Ferritin, Horseradish peroxidase molecules did penetrate the T-system of urea-removal treated toe muscles, as witnessed by the electron-dense deposit in the T-system (Figure 33). Peroxidase itself is not visible in the electron microscope and neither is the reaction product resulting from incubating diaminobenzidine and H_2O_2 with peroxidase (see methods). It has been suggested that the electron-dense material is osmium which has reacted with the reaction products (Reese and Karnovsky, 1967).

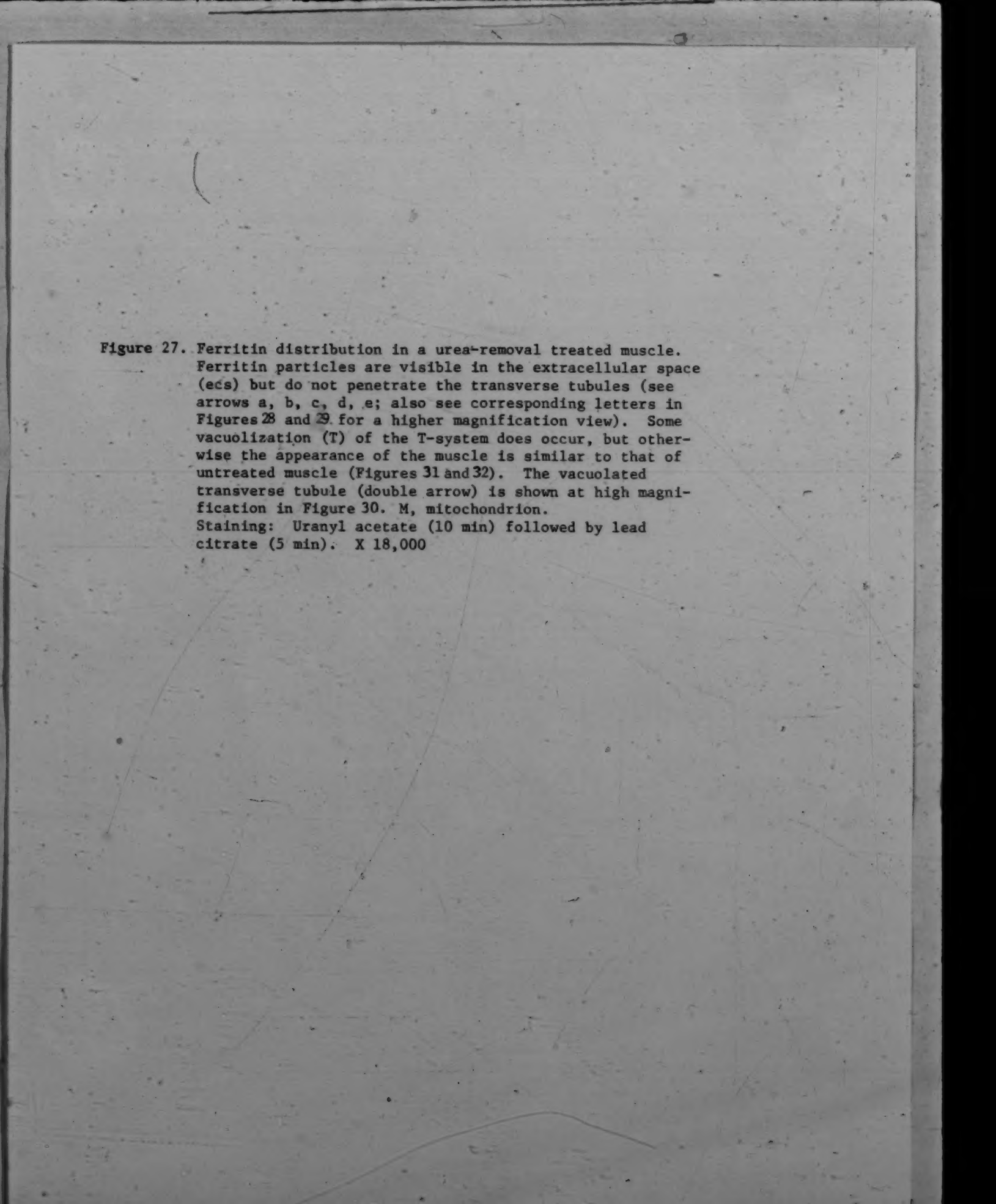
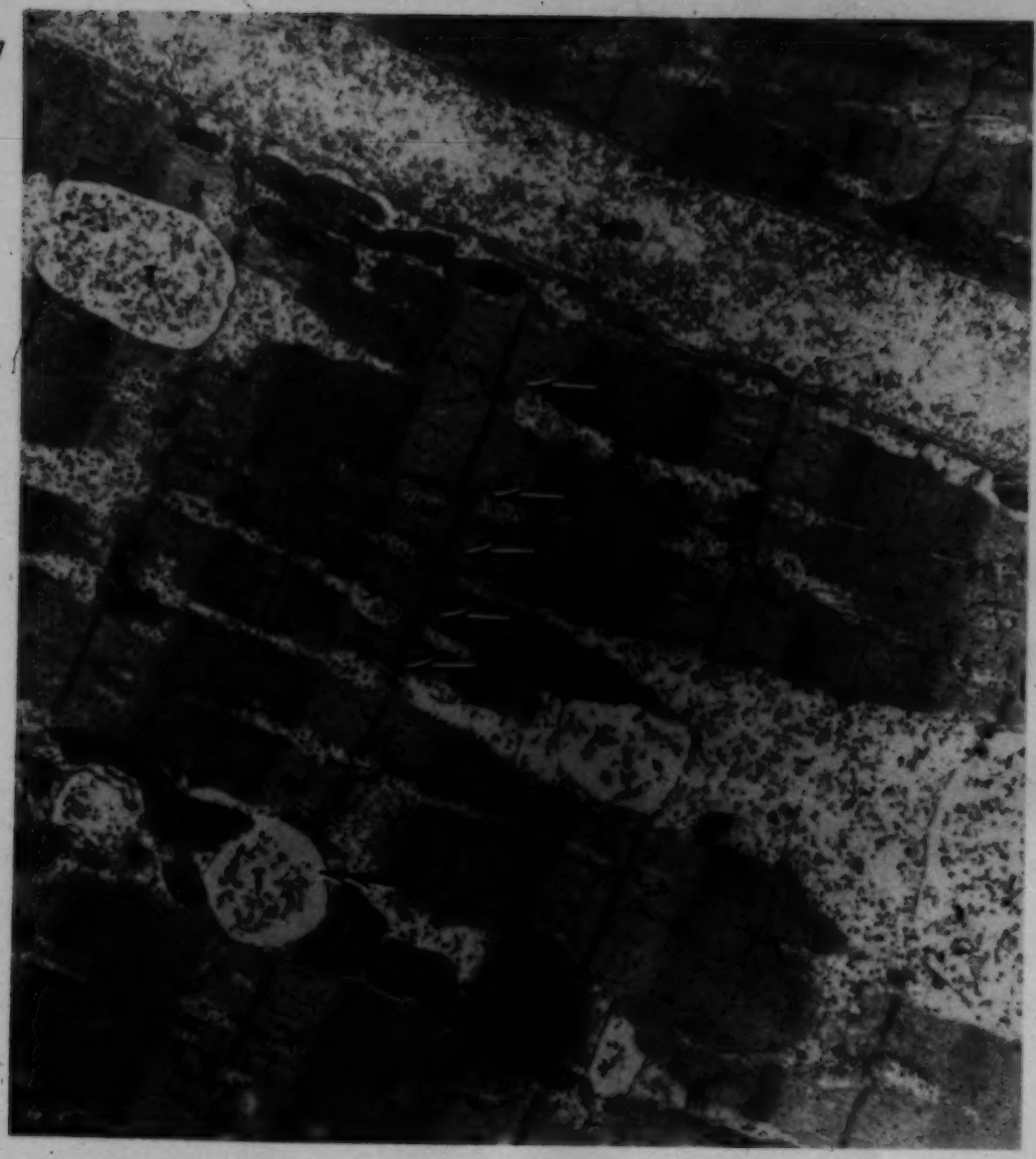


Figure 27. Ferritin distribution in a urea-removal treated muscle. Ferritin particles are visible in the extracellular space (ecs) but do not penetrate the transverse tubules (see arrows a, b, c, d, e; also see corresponding letters in Figures 28 and 29 for a higher magnification view). Some vacuolization (T) of the T-system does occur, but otherwise the appearance of the muscle is similar to that of untreated muscle (Figures 31 and 32). The vacuolated transverse tubule (double arrow) is shown at high magnification in Figure 30. M, mitochondrion.
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 18,000

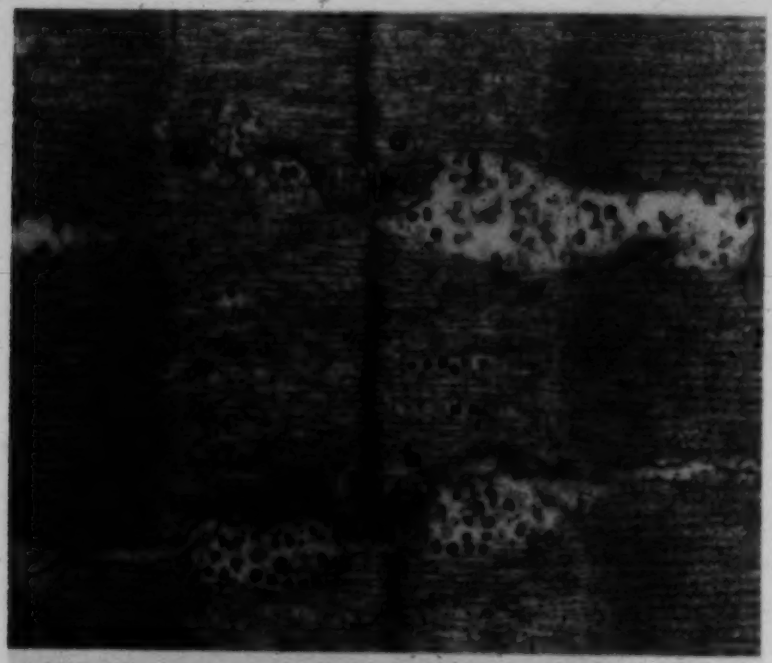
27



Figures 28 and 29.

Triads of urea-removal treated muscle. The triadic sites labelled a, b, c, d, and e are higher magnification views of those in Figure 27. No ferritin can be found in the transverse tubules which appear no different from those in untreated muscle (Figure 32).
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 50,000

28



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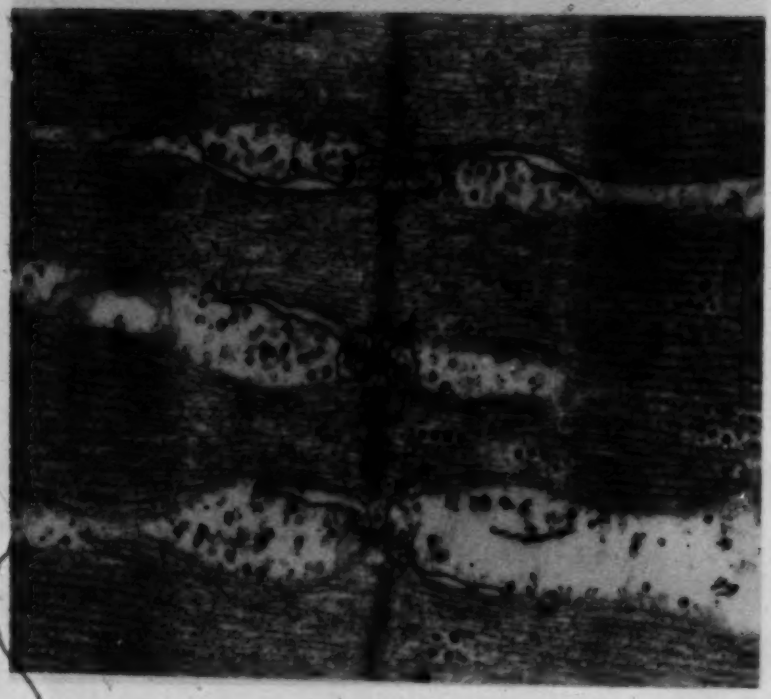

Figure 30. A vacuolated transverse tubule. Swollen segments of the T-system are encountered occasionally in urea-removal treated muscle (Figure 27). The tubular space (T) is flanked by two mitochondria (M). The triad junction separates the tubular space from the terminal cisterna (Tc). Dense particles associated with glycogen granules are found in the tubular space and must have leeched out from the sarcoplasm at places where the tubular membrane was perforated (see arrows). The dense particles are a staining artifact.
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 75,000

30

Figure 31. Ferritin distribution in an untreated muscle. This muscle is the untreated paired counterpart of the muscle depicted in Figure 27. Ferritin particles can be seen in the extracellular space (lower left hand corner) and also in the transverse tubular system (arrows a, b, c, d, e; also see corresponding letters in Figure 32 for a higher magnification view).
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 30,000

31

Figure 32 a, b, c, d, and e

Triads of untreated muscle. The triadic sites labelled a, b, c, d, and e are higher magnification views of those in Figure 31. Note the dense packing of Ferritin particles in the transverse tubules.
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 75,000

32

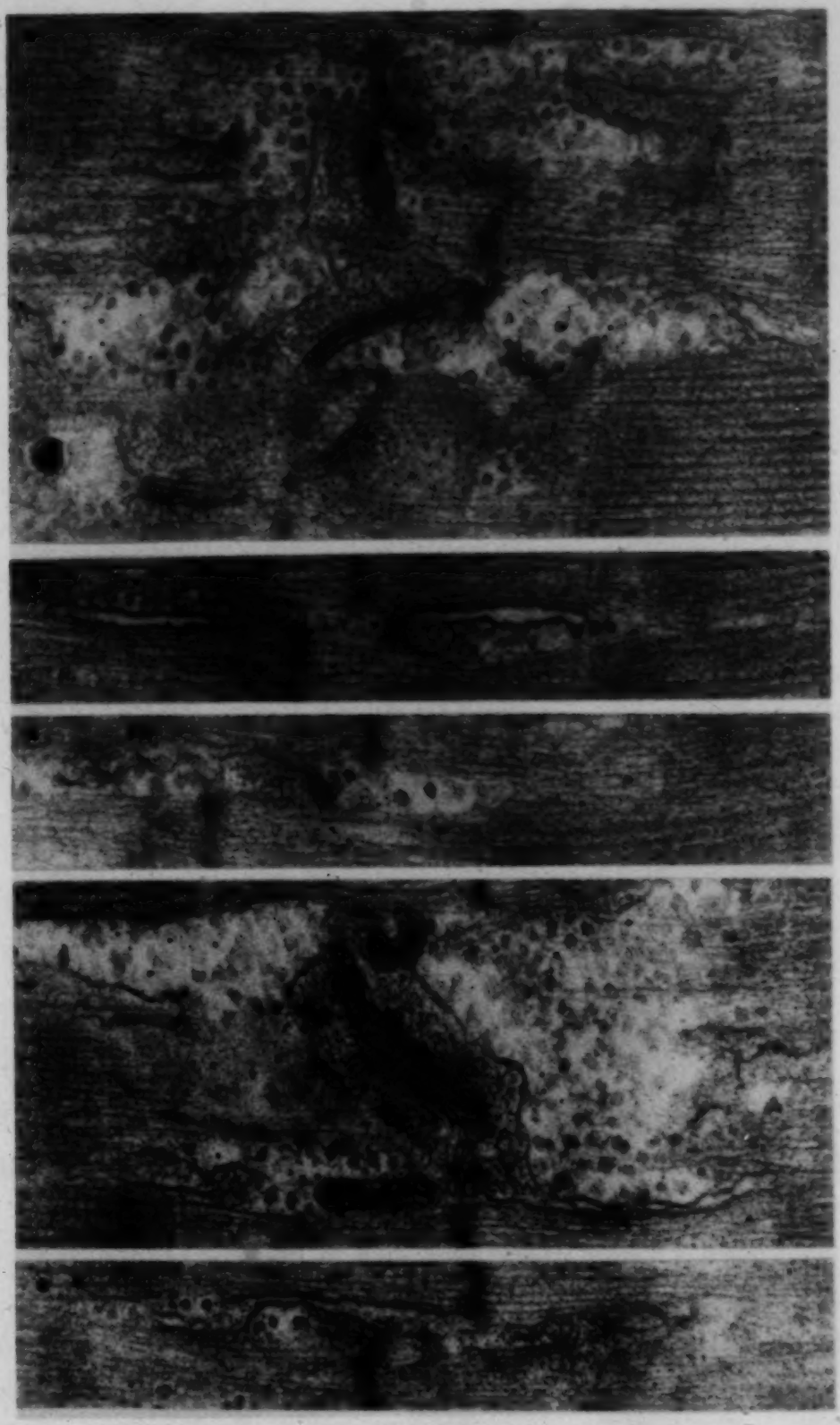

Figure 33. Horseradish peroxidase (Hrp) distribution in urea-removal treated muscle. Peroxidase is present in the T-system (arrows) but its distribution is not as abundant as in untreated muscles (Figure 34). The sarcolemma is densely stained by the Hrp reaction. ecs, extracellular space. M, mitochondrion. T, swollen transverse tubule. F, fat globule.
Staining: Lead citrate (5 min) only. X 14,000

33

Staining of the T-system was not as pronounced as in the untreated control muscle (Figure 34). Higher magnification views of both urea-removal treated (Figure 35) and control muscle (Figure 36) show that staining remained confined to the T-system only.

2.3 Glycerol-removal Treatment.

2.3.1 *Measurements of the triad junction width of glycerol-removal treated toe muscles.* Following identical sampling methods as before (section 2.2.1) micrographs were collected from toe muscles that had been treated with hypertonic glycerol (400 mM) for 10 minutes and were subsequently reimmersed into normal Ringer's solution. At the time when the twitch response was reduced to a minimum this muscle was fixed in a 5% buffered gluteraldehyde together with an untreated muscle from the same frog. In Table 3 measurements of triadic width are summarized. No significant difference between the junctional widths of glycerol-removal treated muscles and their controls was detected. A difference in triadic width between untreated from this batch of frogs (111.2 Å, Table 3) and that of previous experiments (89.1 Å, Table 2) was noted. This discrepancy may have occurred as a result of seasonal variation which is known to occur in amphibians (Dulhunty and Gage, 1973). For example frogs of the first bath (Table 2) were sacrificed in December while the others (Table 3) were sacrificed in August.

2.3.2 *Distribution of Ferritin in glycerol-removal treated frog's skeletal muscle.* Glycerol-removal treated toe muscles were soaked in a Ferritin (20% w/v) Ringer's solution for 1/2 hour follow-

Figure 34. Horseradish peroxidase (Hrp) distribution in untreated muscle. Peroxidase staining is abundant in the T-system. z, Z-line.
Unstained. X 13,000

34
Hrp

Figure 35. A triad of a urea-removal treated muscle. Note that Horseradish peroxidase staining remains restricted to the tubular space only (Hrp). The appearance of the triad is no different from that of an untreated muscle (see below). ecs, extracellular space; z, Z-line; Tc, terminal cisterna. Staining: Lead citrate (5 min) only. X 127,000

Figure 36. A triad of an untreated muscle. Horseradish peroxidase (Hrp) can be seen in the transverse tubule only. Staining: Uranyl acetate (15 min) followed by lead citrate (5 min). X 87,000

35

36

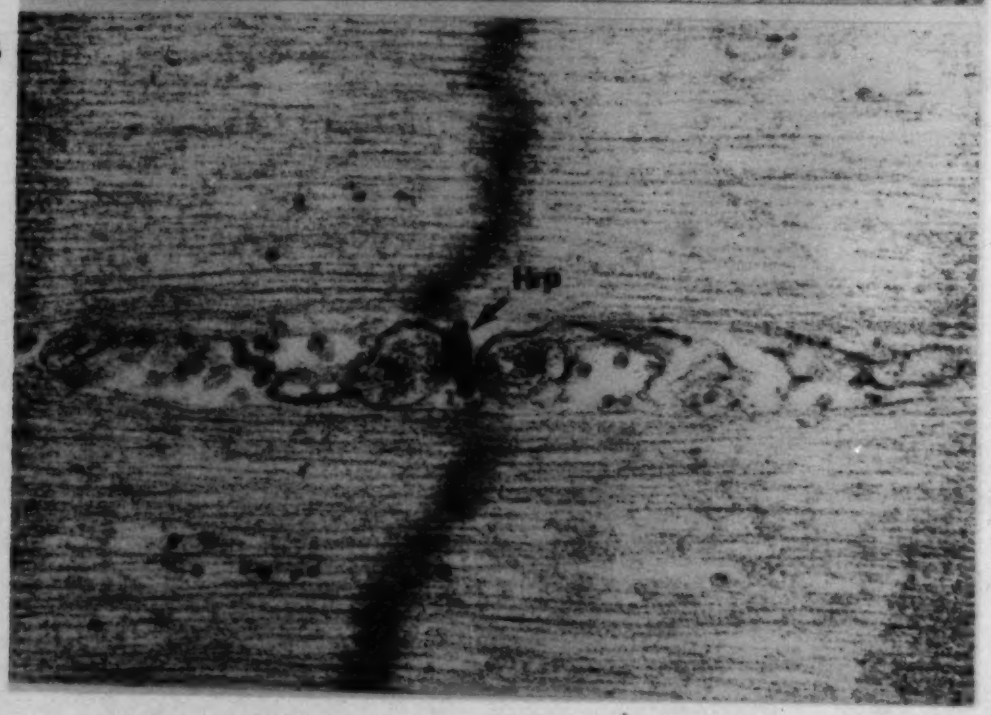

Table 3. Measurements of the width of the triadic junction in untreated toe muscles and in toe muscles subjected to a (10 min) glycerol-removal procedure. The triad junction width listed for each muscle is the mean ± standard error of the mean (SE) of 10 measurements (see Methods).

A. Untreated toe muscles		B. Glycerol-removal treated toe muscles (10 min)	
Muscle no.	Triadic Width (Å)	Muscle No.	Triadic Width (Å)
RC14	108 ± 7	RC13	120 ± 8
RC17	124 ± 4	RC16	104 ± 8
RC19	102 ± 6	RC18	90 ± 5
RC21	116 ± 5	RC20	115 ± 5
RC25	106 ± 5	RC24	99 ± 7
Muscle means ± SE*	111.2 ± 3.9		105.6 ± 5.4

* Triadic width of glycerol-removal (10 min) treated toe muscles did not significantly differ from that of untreated toe muscles. $P>0.05$

ing a 30 minute glycerol-washout period. Untreated muscles (same frog, different leg) were also immersed in a Ferritin-Ringer's solution for 1/2 hour. Both muscles were fixed simultaneously at the end of the Ferritin-soaking period and further processed for electron microscopy (see methods). Figure 37 is a low magnification micrograph of a glycerol-removal treated muscle: note the Ferritin particles in the extracellular fluid and their absence in the transverse tubular system. Figure 38 is a micrograph of an untreated muscle taken at the same magnification as Figure 37. The accumulation of Ferritin is visible in the transverse tubules of the untreated muscle. Figures 39, 40, and 41 are higher magnification views of different triads in an untreated muscle, showing the penetration of Ferritin in the T-system. In contrast Figure 42 illustrates that for glycerol-removal treated muscle, Ferritin remains restricted to the extracellular space only. In an attempt to find out if the T-system remains open to the extracellular space after short glycerol-removal treatments, muscles were subjected to glycerol exposures of 10 and 20 minutes. Apart from the duration of the hypertonic glycerol incubation these muscles and their untreated controls (same frog) underwent the same treatment schedule as described earlier in this section. Some penetration of Ferritin was observed in the 10 minute glycerol-removal treated muscle while no Ferritin was detected in the T-system of the 20 minute glycerol-removal treated muscle. Since the distribution of Ferritin was not consistent throughout the entire T-system of the 10 minute glycerol-removal treated muscles, these experiments were repeated with the smaller molecular weight marker Horseradish peroxidase.

Figure 37. Ferritin distribution in a glycerol-removal treated muscle and its control (inset). Ferritin is present in the extracellular space (ecs) and no ferritin particles can be seen in the T-system (arrows). M, mitochondrion; F, fat globule; SR, sarcoplasmic reticulum in full view.
Staining: Lead citrate (5 min) only. X 30,000

Figure 38. (Inset). Ferritin distribution in an untreated muscle. Ferritin particles are present in the T-system (arrows).
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 30,000

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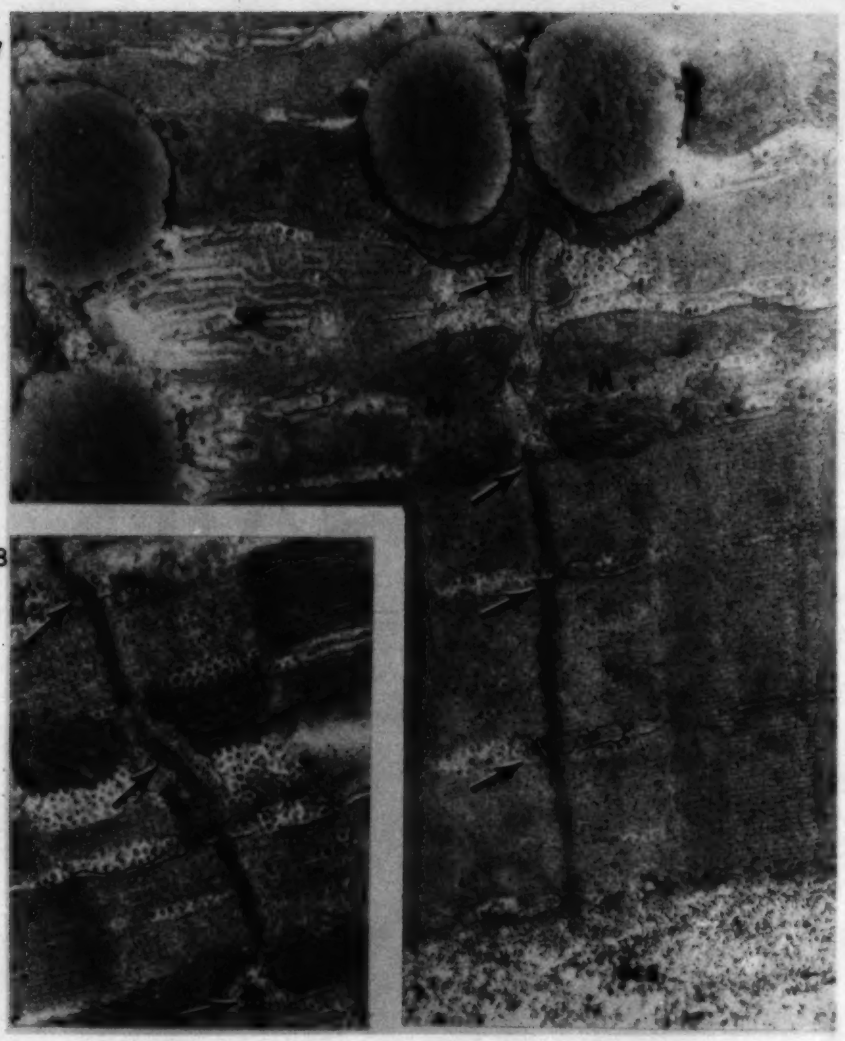

Figure 39. Distribution of Ferritin in untreated muscle. An extended view of the transverse tubule packed with Ferritin particles (arrows). F, fat globule; M, mitochondrion; Tc, terminal cisterna; Z, Z-line.
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 75,000

Figure 40. Distribution of Ferritin in untreated muscle. A different orientation of the triad. Ferritin is present (arrow) in the T-tubule.
Staining: Uranyl acetate (10 min) followed by lead citrate (5 min). X 80,000

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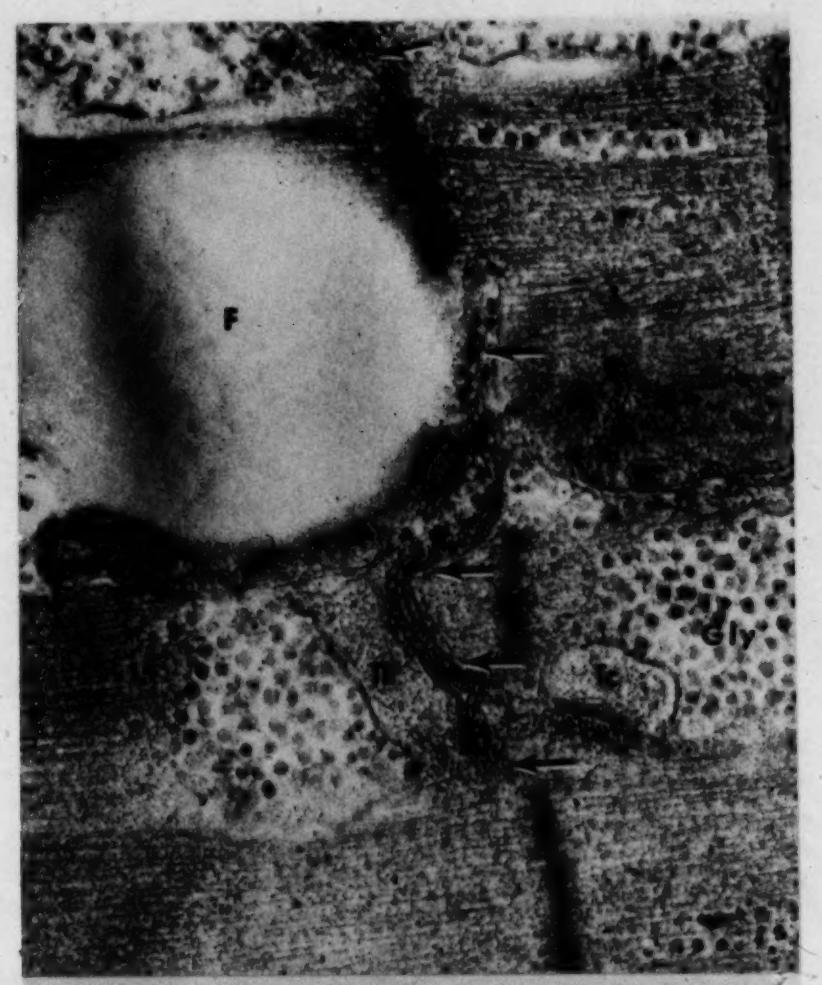

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Figures 41 and 42.

Distribution of Ferritin in untreated (Figure 41) and in (60 min) glycerol-removal treated (Figure 42) muscles of the same frog. Ferritin is present in the extracellular space (ecs) of both muscles. Ferritin accumulates in the T-system of untreated muscle (Figure 41) is excluded from that of glycerol-removal treated muscle (Figure 42). Sl, sarcolemma; V, vacuole; Tc, terminal cisterna; M, mitochondrion.
Staining untreated muscle: Uranyl acetate (10 min) followed by lead citrate (5 min). X 80,000
Staining glycerol-removal treated muscle: Lead citrate (5 min) only. X 75,000

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2.3.3 *Distribution of Horseradish peroxidase in short (10 minute) and long (60 minute) glycerol-removal treated toe muscles.* Muscles incubated in hypertonic glycerol for respectively 10 and 60 minutes were placed in normal Ringer's for 30 minutes. These muscles and their untreated controls (same frog) were subsequently immersed in a Ringer's solution containing Horseradish peroxidase for 30 minutes after which they were further processed for electron microscopy (see methods). Figure 43 is a low magnification view of an untreated muscle whose T-system is intensely stained by the Horseradish peroxidase reaction. Figure 44 offers a higher magnification view of the same muscle and shows 3 triads of which only the transverse tubules are filled with the electron-dense reaction product. No evidence of penetration of Horseradish peroxidase in the T-system of muscles subjected to either a 10 minute of a 60 minute glycerol-removal treatment could be detected (Figures 45 and 46).

2.3.4 *Additional observations in muscles subjected to short (10 minute) and long (60 minute) duration glycerol-removal treatments.* Thus far two morphological features (i.e. width of triadic junction and accessibility of T-system to various markers) were studied in toe muscles. A third morphological parameter was examined to obtain an estimate of the structural integrity of the transverse tubular system. For this purpose the number of 'intact' transverse tubular sites still remaining after glycerol-removal treatments of different duration were compared. An 'intact' tubular site was defined as a triadic structure containing at least one terminal cisterna and a clearly distinguishable transverse tubule. A detailed description of the sampling procedure

Figure 43. Horseradish peroxidase distribution in untreated muscle. This muscle represents a control for the short-glycerol removal treated muscle in Figure 45.
Staining: Lead citrate (5 min). X 15,000

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Figure 44. Triads of untreated muscle stained for Horseradish peroxidase. A high magnification view of triads, showing staining only in the transverse tubules (arrows).
Staining: Lead citrate (10 min) only. X 75,000

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Figure 45. Short (10 min) glycerol-removal treatment and the distribution of Horseradish peroxidase. Muscles were subjected to short (10 min exposure) glycerol-removal treatments. No Horseradish peroxidase was detected in the transverse tubules (arrows). M, mitochondrion; T, swollen transverse tubule.
Staining: Lead citrate (5 min) only. X 12,000

45

Figure 46. Long (60 min) glycerol-removal treatment and the distribution of Horseradish peroxidase. No electron dense staining product could be detected. Note the absence of transverse tubules. M, mitochondrion; Z, Z-line. Staining: Lead citrate (5 min). X 12,000

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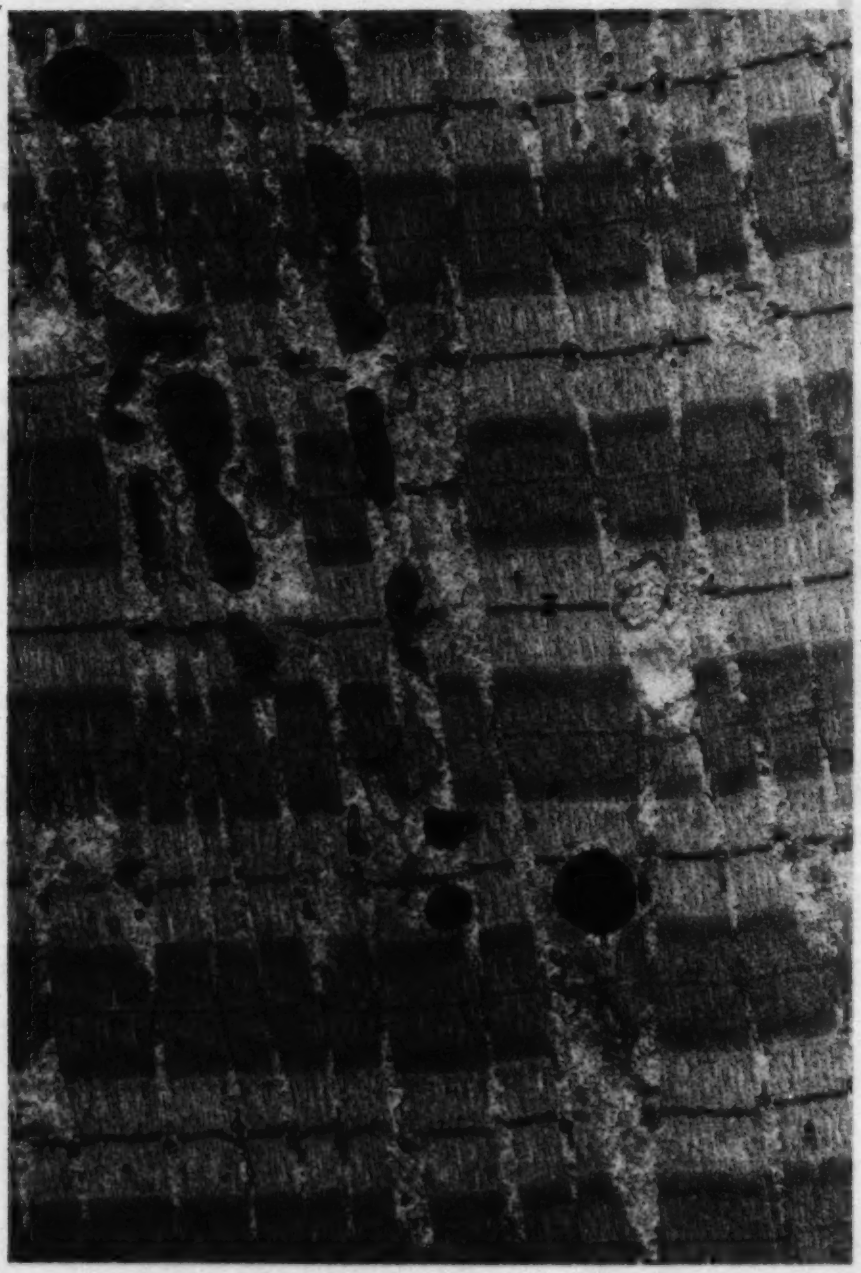

is provided in the methods section (Chapter II, section 6.2). The number of 'intact' transverse tubules was reduced by 60% for 10 minute glycerol-removal treated muscles while a reduction of 77% was observed for the 60 minute glycerol-removal treated muscles (Table 4). No estimation of the structural integrity of the T-system of urea-removal treated muscle fibres was made because the appearance of the individual triads (Figures 28 and 29) was not much different from those of untreated muscle fibres.

3. Electrophysiological Observations

Two types of electrophysiological measurements are often used to determine if the T-system in muscles is still continuous with the extracellular space. These are the late after potential which follows a series of successive action potentials (Figure 47) and the 'creep' in potential produced by prolonged hyperpolarization (Figure 49).

3.1 Late after potentials in urea- and glycerol-removal treated muscles. The slow repolarization that follows a series of action potentials is called the late after potential. The average peak amplitude of the late after potential is 13 mV and its half-time of decay is 270 msec. For measurements only those fibres were selected in which the late after potential was elicited by 10 successive action potentials and in which the resting membrane potentials had not changed by more than 2 mV before and after test recordings. Figure 47A is a typical recording of the late after potential in an untreated muscle. Two pictures of the late after potential were superimposed

Table 4. Estimates of structural integrity of the T-system of glycerol-removal treated toe muscles. Estimates of the average number of intact T-tubular sites (see Methods) were determined in untreated toe muscles (A); toe muscles subjected to 10 minutes glycerol-removal treatment (B); and, toe muscles subjected to 60 minutes glycerol-removal treatment (C). The muscle mean ± standard error of the mean (SE) was determined from measurements in six fibres. (see Methods). The treatment mean ± SE was determined from 4 muscles.

		Average number of 'intact' transverse tubules per 20 myofibrils											
		A. Control				B. Glycerol-removal (10 min)				C. Glycerol-removal (60 min)			
Muscle number		RC17	RC19	RC21	RC25	RC16	RC18	RC20	RC24	RC1	RC3	RC7	RC11
Fibre	1	10.0	8.0	10.7	10.3	6.7	2.3	4.3	3.3	2.0	2.3	5.3	1.0
	2	11.0	9.3	10.3	11.0	5.7	4.7	4.3	3.3	3.0	1.3	3.3	2.7
	3	10.0	7.7	9.7	12.7	3.7	5.3	2.7	1.3	1.7	2.7	5.7	0.7
	4	10.3	7.7	10.0	12.7	5.7	6.0	2.7	5.3	3.0	2.3	2.7	2.0
	5	9.3	9.7	12.3	8.7	3.7	6.3	2.7	1.7	1.0	1.3	4.7	2.7
	6	12.7	11.0	7.7	11.3	4.7	3.3	3.3	7.3	1.7	1.7	1.3	2.0
Muscle means ± SE		10.5 .5	8.9 .5	10.1 .6	11.1 .6	5.0 .5	4.6 .6	3.3 .3	3.7 .9	2.1 .3	1.9 .2	3.8 .7	1.8 .3
Treatment means ± SE*		10.1 ± .4				4.1 ± .5				2.4 ± .5			
Intact T-tubules (%C)		100%				40%				23%			

* The number of intact transverse tubules in glycerol-removal (10 min) treated muscles is significantly different from that of untreated muscles ($P<0.001$). There is a significant difference in the number of intact T-tubules for B and C ($P<0.05$).

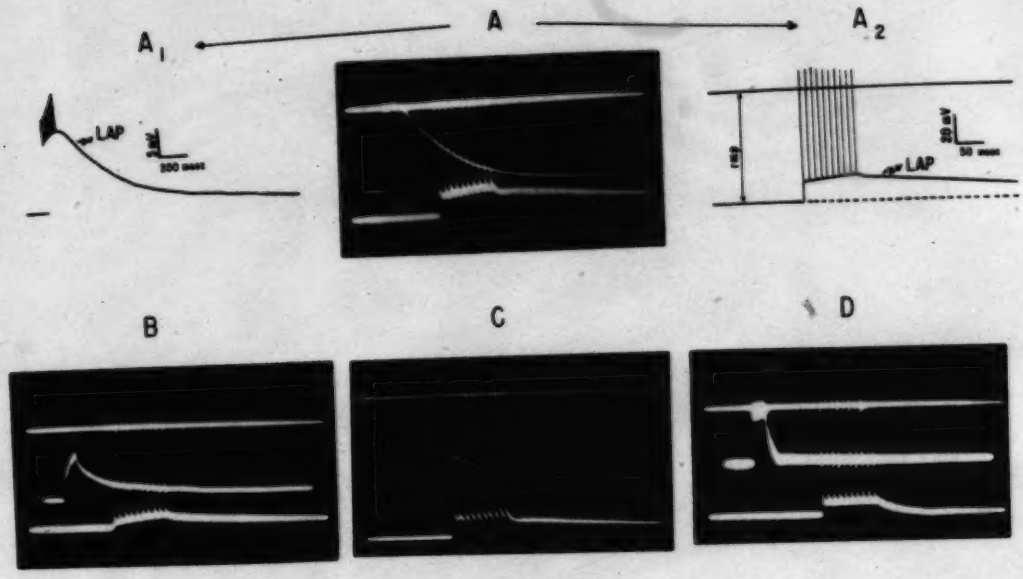

Figure 47. Late after potentials recorded intracellularly from toe muscle fibres under different experimental conditions. A, control; B, urea-removal treatment; C, short (10 min) glycerol-removal treatment; D, long (60 min) glycerol-removal treatment. A_1 and A_2 are tracings obtained from picture A and depict the late after potential (LAP) at slow sweep speed, high gain (A_1) and at faster sweep speed, lower gain (A_2). From tracings such as A_1 information on the decline of the LAP was obtained. Resting membrane potential (rmp) and action potentials were monitored on A_2.

on each other at different gain and different sweep speed in order to examine details of the late after potential and the action potentials simultaneously. Figure 47A$_1$ represents the high gain, slow sweep speed component of Figure 47A, while the same event is displayed at lower gain and faster sweep speed in Figure 47A$_2$. Recordings obtained from urea- or glycerol-removal treated toe muscles were obtained at least 30 minutes after the hypertonic solution had been replaced with an isotonic Ringer's solution; i.e. when the twitch was either abolished or reduced to a minimum (sections 1.1.1 and 1.2.1). Late after potentials could still be elicited in urea-removal treated toe muscles although their amplitude was reduced (Figure 47B). This would indicate that the T-system openings at the surface membrane are still intact. Similar observations were made for muscles subjected to the short duration (10 minutes) glycerol-removal treatment (Figure 47C). The late after potential was absent however in muscles subjected to a long duration (60 minute) glycerol-removal treatment (Figure 47D). These results are summarized in Figure 48 in which the average time course of decline of late after potentials is recorded for the different treatments.

3.2 Creep potentials in urea- and glycerol-removal treated muscles. Creep potentials were recorded from fibres of small sartorius muscles before and 30 minutes after either urea- or glycerol-removal treatment. Figure 49 shows records of potential changes produced by constant current pulses of 1600 msec duration. On prolonged hyperpolarizing current injection into untreated muscle fibres the membrane potential does not reach a steady state but continues to increase

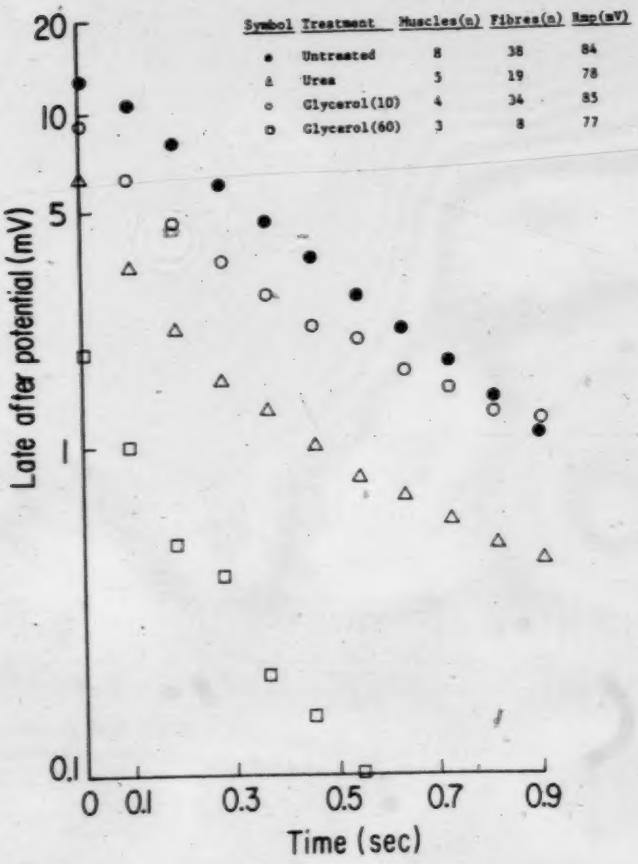

Figure 48. The average decline of the late after potential in toe muscle fibres under different experimental conditions. Measurements were obtained from oscilloscope tracings such as depicted in Figure 1A (also see Methods). The number of measurements to calculate each mean is indicated in the inset above. Resting membrane potentials (rmp) were recorded for the different treatments.

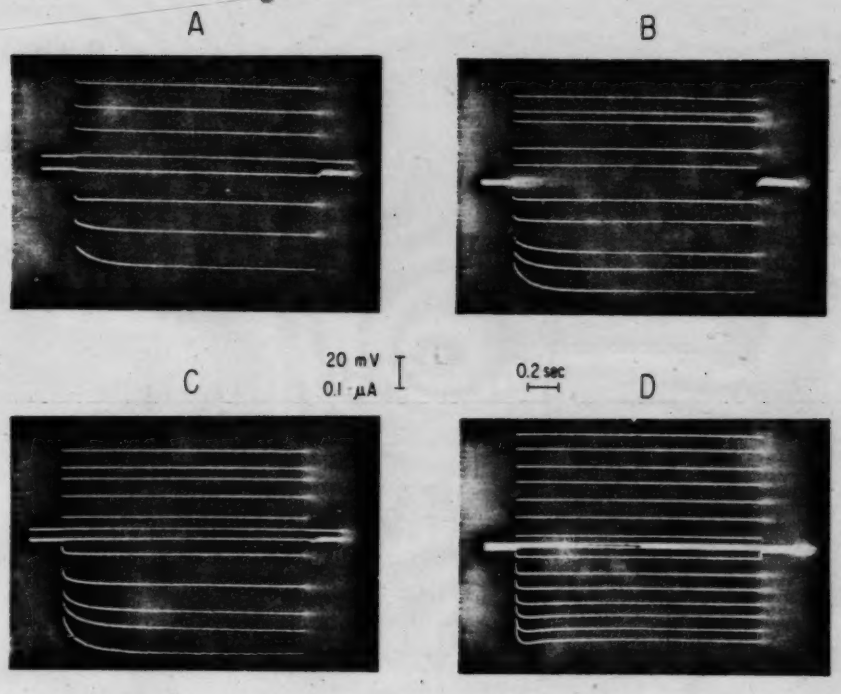

Figure 49. Creep potentials recorded from small sartorius muscle fibres under different experimental conditions. A, control; B, control; C, urea-removal treatment; D, glycerol (60 min) removal treatment. C and D were recorded 30 min after the muscles were returned to Ringer's solution.

slowly in the hyperpolarizing direction (Figures 49 A and B). This slow hyperpolarization is usually referred to as the 'creep' potential and is caused by a decrease in membrane conductance resulting from declining K^+-concentrations in the T-system (see Chapter I, section 5). The creep potential is also plotted graphically (Figures 50, 51) by measuring the membrane displacement of 75 msec and again at 1350 msec after the onset of the stimulating current. The increment in membrane potential within this time interval is illustrated by a shift in the current-voltage relation towards the hyperpolarizing direction and is thought to represent the creep potential. Current-voltage relations obtained in this fashion indicate that for untreated muscles (Figures 50 and 51) creep potentials become noticable at membrane potentials more negative than -100 mV. Similarly the creep potential can still be observed in urea-removal treated muscles (Figures 49C, 50), but glycerol-removal treatment does abolish the creep potential (Figures 49D, 51).

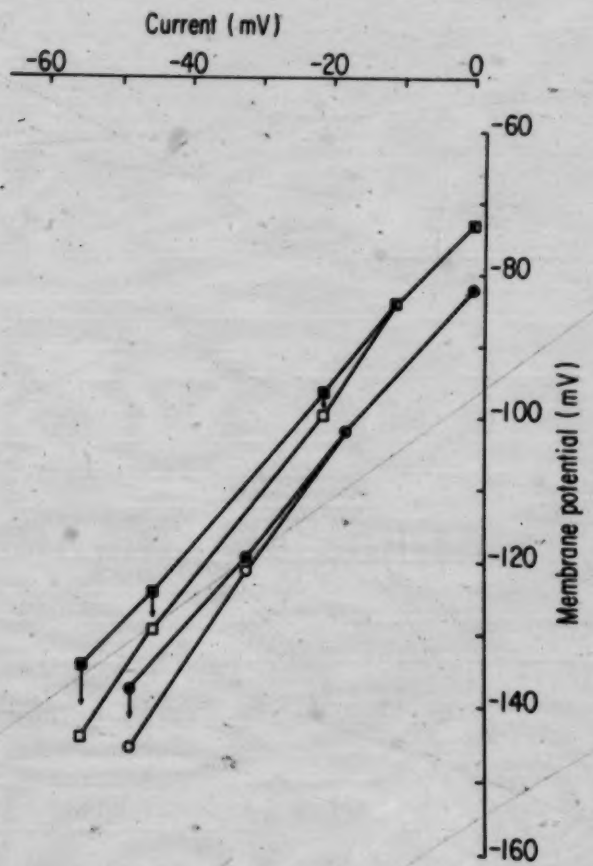

Figure 50. Current-voltage relations for a urea-removal treated muscle fibre. Measurements of membrane displacement (see Figure 3C) were made at 75 msec (●, ■) and at 1350 msec (o, □) after the onset of the stimulating current. (o, ●); untreated muscle. (□ , ■), urea-removal treated muscle fibre. Creep is represented by arrows pointing downward. Stimulating current is recorded as the voltage drop across a 100 K resistor.

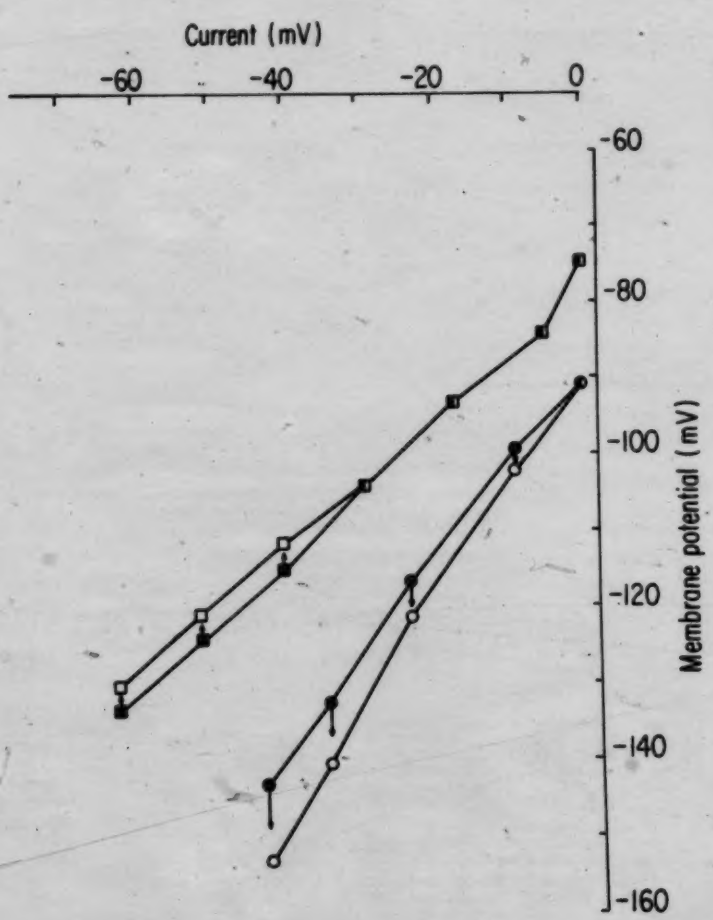

Figure 51. Current-voltage relations for a glycerol-removal treated muscle fibre. Measurements of membrane displacement (Figure 3D) were made at 75 msec (●, ■) and at 1350 msec (o, □) after onset of the stimulating current. (o, ●), untreated muscle; (□, ■), glycerol removal treated muscle. Creep is represented by arrows pointing downward. Stimulating current is recorded as the voltage drop across a 100 K resistor.

CHAPTER IV. DISCUSSION

Hypertonic solutions, prepared by adding large amounts of NaCl or sucrose to a Ringer's solution selectively inhibit the twitch or potassium contracture of frog muscle fibres without impairing the conduction of action potentials or the development of contractures induced by caffeine (Hodgkin and Horowicz, 1957; Howarth, 1958; Caputo, 1966). This hypertonic condition is occasionally utilized in microelectrode studies of action potentials in order to eliminate muscle movement.

When non-electrolyte substances such as glycerol or urea were used as osmotic agents, it was found that these substances affected the contractile response of frog's skeletal muscle in quite a different fashion (Fujino et al., 1961; Yamaguchi, et al., 1962). The twitch response was only transiently inhibited and then recovered during the exposure to the hypertonic media. Fujino et al. (1961) called this phenomenon 'the glycerol effect', because they found it highly specific for glycerol. These workers also noted another phenomenon to which they did not attach much importance although it later became an essential tool in the investigation of e-c coupling processes in fast skeletal muscle. They reported that reimmersion of muscle fibres in an isotonic Ringer's solution after a hypertonic exposure to glycerol, resulted in an irreversible loss of the twitch response. Six years later Howell and Jenden (1967) reinvestigated this phenomenon and attributed the loss of mechanical activity to a disruptive effect on the T-system causing a closure of the T-tubules at their openings with the surface of the muscle fibre. In order

to distinguish this effect from the 'glycerol effect' I have called it the 'glycerol-removal effect' in this thesis. Caputo (1968) found that the glycerol-removal effect could be achieved with a number of other non-electrolytes such as urea, ethylene glycol, propylene glycol and acetamide and concluded that the resultant changes in mechanical activity brought about by these non-electrolyte substances must have been related to their membrane-penetrating characteristics.

Other workers (Fujino, 1972; Oota and Nagai, 1973) have examined in greater detail the effects of a urea-removal treatment in frog skeletal muscle and observed a loss of twitch response which they attributed to an interruption of e-c coupling. In addition Oota and Nagai (1973) demonstrated that unlike glycerol, the urea-removal treatment did not block e-c coupling by a closure of the T-tubules at their surface openings, since the extracellular marker molecule Ferritin was still shown to enter the T-system under these conditions. Instead the block in e-c coupling following urea-removal was attributed to an increase in the width of the triad junction.

1. Urea-removal

I have further investigated the ultrastructural changes that occur as a result of a urea-removal treatment and was able to confirm Oota and Nagai's findings (1973), that under these conditions the triad junctional width was increased (Table 2). Contrary to Oota and Nagai's observations, in the present experiments Ferritin was excluded from the T-system (Figures 27, 28, 29). However, subsequent electronmicroscopic studies demonstrated that Horseradish peroxidase, whose molecular diameter is smaller than that of Ferritin, did penetrate the T-system

(Figures 33 and 35). It was concluded therefore that the transverse tubules in urea-removal treated toe muscles are communicating freely with the extracellular space although its surface openings may be somewhat constricted. The observed difference in Ferritin distribution between our experiments and those of Oota and Nagai (1973) may be due to a species difference. Apart from the occasional formation of vacuoles no other obvious structural irregularities were observed.

The structural integrity of the individual triads appeared normal (Figures 28 and 29) except for the measured increase in the width of the triad junction (Table 2). Additional support for the intactness of the T-system is provided by the electrophysiological observations that the late after potential (Figures 47B and 48) and the creep potential (Figures 49C and 50) were present in urea-removal treated muscle fibres.

Some loss of normal T-tubular functioning however is indicated by the reduction in amplitude and duration of the late after potential and could well be due to a reduction in T-tubular volume. It is well known (Freygang et al., 1964; Kirsch, 1977) that the late after potential is caused by the accumulation in and the subsequent delayed outward diffusion of K^+-ions out of the T-system during and following a train of successive action potentials. Reduction of the T-tubular volume would thus be expected to reduce the total quantity of K^+-ions deposited in the T-system and also the diffusion path for these ions. It is tempting to postulate, in addition, that some transverse tubules become 'pinched off' beyond their vacuolated segments. The mechanism of this 'pinching off' process would be similar to that discussed below for glycerol-removal treated muscles although

the frequency with which this occurs would be much lower in urea-removal treated muscles. Such 'pinched off' transverse tubules would thus become isolated from the surface membrane and no longer participate in the genesis of the late after potential. The only evidence we have to support this possibility is the observation that membrane lesions in the vacuolated area of the T-system do occur (Figure 30) and that a drop in the resting membrane potential of urea-removal treated fibres was observed (Figure 48, insert) indicating the possibility of a 'pinching off' and resealing process in the transverse tubules.

That the loss of twitch in urea-removal treated muscles is caused by an impairment in e-c coupling is obvious since action potentials could still be recorded (Figure 47B) and contractures could still be elicited by caffeine (Figure 9). Consistent with our experimental data is the suggestion (Oota and Nagai, 1973) that the block in e-c coupling is caused mainly by an increase in triadic junction width but some detubulation may also contribute to the observed e-c coupling block.

The degree of participation of these different structural changes in the impairment of e-c coupling will depend on the type of muscle subjected to a urea-removal treatment. For instance, in frog sartorius muscle we have evidence (Figure 19) which suggests that the increase in triadic width may not be the main factor that causes the e-c coupling loss following a urea-removal treatment. Because the frog sartorius muscle contains a greater proportion of large diameter fibres than the toe muscle, the average length of the transverse

tubular system per fibre is increased accordingly. A longer T-tubular diffusion path may lead to more intense vacuolization and detubulation in these large diameter muscle fibres during the washout of hypertonic urea.

Our present view of the events occurring during e-c coupling in vertebrate skeletal muscle may be summarized as follows: activation of the intracellular contractile proteins is produced by a sudden increase in the myoplasmic calcium ion concentration. Most of this calcium comes from the sarcoplasmic reticulum where it is stored in the resting fibre (Sandow, 1965). Depolarization of the transverse tubular membrane is required to cause the release of calcium from the sarcoplasmic reticulum (Gage and Eisenberg, 1967). However, the mechanism by which T-tubular depolarization produces the release of calcium from the SR remains in doubt. In the past, two alternative hypotheses have received the greatest experimental interest: (1) that current flows from T-tubules through the SR membranes causing calcium release (Chapter 1, section 3.1) and, (2) that "trigger calcium ions" are released into the triadic junction, diffuse to the SR membrane and act on the SR membranes to stimulate calcium release (Chapter 1, section 3.3). Recently a third hypothesis that voltage dependent charge movements in the T-tubular membrane control the release of SR calcium was added to this list (Chapter 1, section 3.2). In view of the current controversy surrounding the involvement of calcium in e-c coupling, the urea-removal treated toe muscle represents a suitable preparation for testing this involvement of calcium, because in this preparation much of the T-system is freely accessible from the extracellular space. Therefore increasing the external calcium ion

concentration should directly exert its effect on the widened triad junction. Assuming that calcium ions are involved in the coupling between T-tubular depolarization and the release of SR-calcium, it was anticipated that increasing the extracellular Ca-ion concentration would antagonize the e-c coupling block in one of two possible ways: (1) by decreasing the width of the triad junction, or (2) by increasing the amount of trigger calcium released into the triadic junction with each action potential.

It was observed that placing a toe muscle in Ringer's solution with an elevated (5 mM) calcium concentration following urea-removal treatment prevented the complete block of e-c coupling normally produced when using Ringer's with 1.08 mM calcium (Figures 12 and 13). Since the increase in width of the triad junction was neither prevented nor reduced under the conditions of high calcium (Table 2) the possibility that the antagonism of the e-c coupling block was due to an effect on the triad junction width was ruled out. The alternative explanation for the antagonism of the e-c coupling block would thus be that the increase in extracellular calcium ion concentration increases the amount of trigger calcium that enters the triad junctional space with each action potential.

It is known that in frog's skeletal muscle action potentials give rise to a transient influx of calcium ions (Bianchi and Shanes, 1959). The greater part of this influx must take place across the membranes of the T-system because its surface area amounts to approximately 85% of the total external membrane area of a muscle fibre (Peachey, 1965). When an action potential reaches the T-tubules,

calcium ions are released into the sarcoplasm and into the triad junction where these ions interact with the membranes of the terminal cisternae to cause the release of calcium required for mechanical activation. In urea-removal treated muscle the increase of triad junctional width would reduce the amount of calcium ions reaching the terminal cisternal membranes due to the following factors: (1) a decrease in calcium concentration due to an increase in triadic junction volume, and (2) an increased diffusion of calcium out of the triad junction into the sarcoplasm. Increasing the concentration of extracellular calcium ions therefore would be expected to compensate to some degree for the two factors causing a decrease in the amount of calcium ions reaching the terminal cisternae.

It is quite possible that a widening of the triad junction could also be the cause of a block in e-c coupling for the two alternative models of e-c coupling. In the case of the current-coupling model, the increased triadic width would increase the resistance to current flow across the triad junction and thereby would decrease the amount of current reaching the terminal cisternae. In case of Chandler's model (1976b) for e-c coupling an increase in triadic width could very well interfere with the postulated mechanical linkage extending across the triad junction or else might interfere with the charge movements in the T-tubular membrane. It would be unlikely however to explain the calcium antagonism of the e-c coupling block on the basis of either of these two models.

Thus far our findings support the general prediction that the calcium antagonism of the e-c coupling block in urea-removal

treated toe muscles may be associated with an enhancement of the calcium influx occurring with each action potential. Our experimental results do not allow us to determine exactly how the total amount of trigger-calcium may be increased following an elevation of the extracellular calcium-ion concentration. The following 3 mechanisms by which this may occur are discussed below. Firstly, it is possible that the influx of extracellular calcium ions into the triad junction during an action potential may be increased due to the increased concentration gradient for calcium-ions across the T-tubular membranes. Not consistent with this possibility is the experimental work of Bianchi and Shanes (1959) who suggested that the transient calcium influx during a membrane depolarization represents the translocation of surface membrane-bound calcium rather than in inward flux of extracellular calcium ions across the sarcolemma. In keeping with earlier evidence, Bianchi (1969) proposed a more detailed model of the events occurring during the coupling step between excitation and contraction of muscle fibres. He postulated that calcium bound at the outer aspect of the T-tubular membrane acts as a stabilizing factor for the release of trigger-calcium which was bound to the inner aspect of the T-tubular membrane. During the process of depolarization this stabilizing-Ca^{++} is displaced which in turn allows trigger-Ca^{++} to be released into the triadic junction. The trigger-Ca^{++} sites can then only be reoccupied if the membrane repolarizes and if extracellular Ca^{++} is present in the T-tubular lumen to saturate the stabilizing sites. The second mechanism by which the amount of trigger-calcium may be increased under conditions of high extracellular

calcium is in line with Bianchi's model of e-c coupling (1969) and would involve an increase in the number of stabilizing-calcium sites saturated with calcium. This in turn would increase the amount of trigger-calcium available for release. It is unlikely however that high (5 mM) extracellular calcium could occupy more stabilizing-sites because the calcium-binding sites on the muscle fibre membranes were found to be fully saturated at external calcium concentrations from 0.3 to 1.0 mM (Bianchi and Shanes, 1960). Thirdly, high extracellular calcium may simply increase the passive influx of calcium into muscle fibres. The resulting increase in myoplasmic free calcium, provided its concentration remains below that for the contraction threshold, would increase the total amount of calcium interacting with the terminal sacs during a given action potential and thus counteract the effects of an increased triad junctional width on the e-c coupling process. The elevation of myoplasmic calcium ion concentration would be similar to that produced with subthreshold concentrations of caffeine (Bianchi, 1968) or potassium (Vos and Frank, 1972).

Although this third mechanism may well explain the calcium antagonism of the loss of e-c coupling in urea-removal treated muscle fibres, it is not consistent with the observation that in untreated muscles high calcium slightly reduces the twitch tension (Figure 14). It has been suggested that the effect of increasing the extracellular calcium ion concentration in untreated muscles is similar to that produced with high concentrations of other multivalent cations such as Zn^{++}, Cd^{++}, Mg^{++} (Parry et al., 1974), Mn^{++} (Chiarandini and Stefani, 1973; Sakai et al., 1974), or La^{+++} (Weiss, 1970; Parry et al., 1974),

all of which reduce the twitch tension. These multivalent cations are thought to replace stabilizing calcium from their membrane sites in the T-system causing trigger-calcium to be bound more tightly and thus prevent their release when muscles are stimulated. In order to reconcile the two unrelated effects of high calcium on e-c coupling, as discussed above, we have considered the possibility that these effects may operate simultaneously and that the expression of each of these effects depends on the experimental condition to which a muscle is subjected.

Frank (1962, 1978a) observed that cobalt-ions were able to prevent the loss of the mechanical response of muscles placed in a zero-calcium solution. It is possible that this effect is due to a stronger binding of trigger calcium to its membrane sites thereby slowing down the loss of Ca-ions by diffusion into the 0-Ca solution in the T-tubule lumen. In order to determine if increased binding of trigger calcium to its release sites also plays a role in the observed calcium-antagonism of the twitch loss in urea-removal treated muscle, the effects of Co^{++} were tested in this preparation. Unlike high calcium, cobalt-ions did not partially prevent the loss of twitch following a urea-removal treatment (Figure 18). Thus assuming that cobalt-ions act to increase the membrane binding of trigger-calcium ions the above results imply that this effect does not play a role in the calcium-antagonism of the twitch loss in muscles subjected to a urea-removal treatment. In support of this interpretation is the experimental finding (Figure 17) that a phosphate-buffered Ringer's solution, which has also been shown to increase the binding of trigger-calcium to its membrane (Frank, 1978b), does not antagonize the loss

of twitch in muscles subjected to a urea-removal treatment. The absence of an effect of cobalt-ions on the urea-removal treatment induced loss of the twitch also would indicate that these cations do not act in a manner analogous to trigger-calcium, nor is it likely that Co^{++} is able to potentiate the influx of trigger calcium during an action potential.

In conclusion, the experimental data in this thesis together with the available evidence from the literature seem to suggest that of the possible mechanisms discussed above, the third is the one most likely to play a role in the calcium antagonism of the loss of e-c coupling in muscles subjected to a urea-removal treatment. By this mechanism the elevation of the extracellular calcium-ion concentration would enhance the passive influx of calcium into the muscle fibres. The resulting increase in the concentration of myoplasmic free calcium would in turn increase the total amount of calcium interacting with the terminal cisternae during an action potential and would oppose the effects of an increase in triad junctional width on the e-c coupling process.

2. Glycerol-removal

The transient nature of twitch tension reduction and contracture response during an exposure to hypertonic glycerol-Ringer's solution i.e. 'glycerol effect' (Figure 20A), was similar to that which occurs in a hypertonic urea-Ringer's solution (Figure 8). The initial contracture response and inhibition of twitch tension are associated with rapid outward water movements that occur in response to the increased tonicity of the bathing medium of the muscle. These osmotic water movements have been monitored as a sudden decline in fibre volume (Krolenko and Adamjan, 1967; Caputo, 1968) and have been observed also in muscles exposed to solutions made hypertonic with other solutes, i.e. sucrose and NaCl, to which the membrane is relatively impermeable.

It is thought that the sudden shrinkage in fibre volume which ensues the outward water movements, gives rise to a perturbation of the SR membrane system and that this in turn causes the release of SR calcium which produces a contracture response. The simultaneous inhibition of twitch response may very well be caused by the increased osmolarity of the sarcoplasm incurred by the loss of intracellular water. The transitory nature of the reduction in twitch tension during the glycerol incubation (glycerol effect) is a feature only produced by substances which have a high membrane permeability. In the case of hypertonic glycerol exposures the initial outward water movements were possible only because the glycerol influx across the membrane lags behind that of the faster movement of H_2O. As soon as glycerol penetrates the membrane, water moves in with it and the osmotic difference across the membrane decreases and the cell volume

increases, resulting in a recovery of twitch tension. From the recovery rate of the twitch tension, it is clear that glycerol must penetrate the muscle fibre membrane at a faster rate than urea. It is unlikely that urea or glycerol, at least at the concentrations we have employed, permanently impair the normal functioning of a muscle fibre because the ability to twitch recovers during the exposure to these chemicals (Figures 8 and 20A) while action potentials and resting membrane potentials remain virtually unaltered (Fujino et al., 1961; Oota and Nagai, 1973). A recent paper by Boethius and Rydqvist (1977) reports no changes in passive electrical properties of muscles exposed to urea concentrations as high as 1 M.

When, following a one hour glycerol (400 mM) exposure, muscles are reimmersed in an isotonic glycerol-free Ringer's solution the twitches are abolished irreversably (Howell and Jenden, 1967; Howell, 1969). I have referred to this treatment procedure as the glycerol-removal treatment and have observed mechanical effects induced by such a treatment similar to those previously reported with one exception; the twitch response was never completely abolished but was reduced to 10% of pre-glycerol twitch tensions. According to the literature, a complete inhibition of the twitch response can only be obtained if either longer glycerol exposure times (Nakajima et al., 1973) or greater concentrations of glycerol (Dulhunty and Gage, 1973b) are employed.

It is now known that glycerol-removal treatment abolishes or greatly reduces the coupling between the action potential and the muscle twitch (Howell, 1969; Gage and Eisenberg, 1969b). That this is so can be demonstrated by the fact that action potentials can still be

elicited (Figure 47D) and that caffeine can still elicit a mechanical response (Figure 21). Originally it was suggested that the main structural change associated with these changes in the e-c coupling process was a closure of the T-tubules at their surface openings (Krolenko, 1969; Eisenberg and Eisenberg, 1968) followed by a general disruptive effect on the T-tubules themselves (Howell, 1969). The closure and subsequent disruption of the T-system can be attributed to osmotic water fluxes and swelling of the transverse tubules following the washout of the hypertonic glycerol solution. This phenomenon will be discussed later.

The results obtained in the present study have confirmed earlier EM studies (Eisenberg and Eisenberg, 1968; Krolenko, 1969; Nakajima et al., 1969, 1973) and have shown that extracellular marker molecules such as Ferritin (Figures 37 and 42) and HRP (Figure 46) do not enter the tubular space of (60 min) glycerol-removal treated muscle fibres. Moreover the late after potential (Figure 47D) and the creep (Figure 49D), both of which are associated with normal functioning of the T-system, were abolished. In addition glycerol-removal treatment was shown to have a considerable disruptive effect on the structural integrity of the triads (Table 4C). These experimental results strongly support the original notion (Howell, 1969) that 'detubulation' of the T-system constitutes the anatomical effect responsible for the interruption of e-c coupling in glycerol-removal treated muscles.

Dulhunty and Gage (1973a) brought to light an interesting observation: they noted that a short exposure of muscles to a hypertonic glycerol solution followed by a reimmersion in a glycerol-free

Ringer's solution (i.e. a short glycerol-removal treatment) also abolished the twitch tension. In contrast to the conventional 60 minute glycerol-removal treatment, no reduction in membrane capacitance was observed in short glycerol-removal treated muscles, indicating that the transverse tubular system was still intact and freely accessible from the extracellular space. These observations led Dulhunty and Gage (1973a) to the conclusion that instead of detubulation, the main event responsible for the e-c coupling block in glycerol-removal treated muscle fibres is stress on the triad junction caused by initial outward movements of glycerol and H_2O from the sarcoplasm into the transverse tubules. They attrubuted the larger swelling and eventual closure of the T-system (i.e. detubulation) to the much slower efflux of glycerol and H_2O from the sarcoplasmic reticulum into the T-tubules.

The nature of the e-c coupling impairment during short glycerol-removal treatments was investigated here. In particular it was of interest to determine if the shorter exposure time to glycerol during the treatment was associated with a widening of the triad junction similar to that observed for urea-removal treated muscles.

In an attempt to distinguish a possible structural effect on the triad junction from the general detubulation effect, muscles were subjected to glycerol-removal treatment of varying glycerol exposure times. According to earlier reports (Dulhunty and Gage, 1973a) glycerol-removal treatments with short glycerol exposure times were found to leave the continuity of the transverse tubules with the extracellular space intact. With progressively

longer glycerol exposures the swelling of the tubular system may be expected to increase until the surface openings of the transverse tubules coalesce and the T-tubules are sealed off from the extracellular space. At that point any other effect on the triad junction would be overshadowed by the detubulation effect. The effect of elevated Ca^{++}-ion concentrations on the reduction in e-c coupling following glycerol-removal treatments of various glycerol exposures was studied in sartorius (Figure 23) and toe muscles (Figure 22) of the frog. It was found that a five-fold increase in the extracellular calcium-ion concentration did not antagonize the reduction of e-c coupling induced by short glycerol-removal treatments. There seems to be a trend, especially after a 10 or 20 minute glycerol exposure (Figures 22A and B) for calcium to exert a slight antagonism of the reduction in e-c coupling, however the difference was found to be insignificant. It may have been possible however that a longer recovery period in elevated Ca^{++} Ringer's solution, i.e. > 35 min, following the 10 and 20 minute glycerol exposures would have shown a calcium related antagonism of the reduction in e-c coupling. This is indicated by the findings of Bianchi and Bolton (1974) who showed that glycerol-removal treated muscles undergo an 8-fold increase in ^{45}Ca-influx which disappears after prolonged recovery in Ringer's solution. The possibility that a longer recovery period of glycerol-removal treated muscles may be required to stabilize the sarcolemmal membrane and hence to demonstrate an antagonizing action of calcium remains to be explored.

The absence of an antagonizing effect of calcium indicates that the structural or functional changes responsible for the reduction

of e-c coupling after short glycerol-removal treatments are not like those for the urea-removal treatment. More direct evidence in support of this conclusion was provided by ultrastructural studies in which the width of the triad junction were measured. No difference in triad width was observed between untreated and 10 min glycerol-removal treated muscles (Table 3). An interesting finding which emerged during the above studies was that the twitch reduction after glycerol-removal treatment seemed independent of the duration of the glycerol exposure up to 60 min. This in contrast to urea-removal treatments where a definite relationship between urea exposure (i.e. 'load time') and twitch tension reduction was shown to exist (Figure 11). The gradual reduction of e-c coupling with increasing 'load times' of urea can be explained by a progressive increase in triad width as discussed earlier. On the other hand the rapid reduction in e-c coupling incurred with glycerol-removal treatment independent of its load times seems to suggest again a different mechanism of action. This led us to reinvestigate the functioning of the T-tubular system after short (10 min) glycerol-removal treatments. To our surprise and contrary to the predictions made by Dulhunty and Gage (1973a) electron microscopic studies with the extracellular marker Horseradish peroxidase showed that these molecules did not enter the transverse tubules (Figure 45). Microelectrode studies however have indicated that the functioning of the T-tubular system was not impaired, since undiminished late after potentials could be recorded from short exposure glycerol-removal treated muscle fibres (Figure 47C). In agreement with these findings is the interpretation that the surface openings of the transverse

tubules are constricted to such an extent that the passage of Horseradish peroxidase whose moledular diameter is approximately 60 Å is prevented, while smaller molecules and ions can still move in and out of the T-system. The decline in the number of intact triads (Table 4B) may account for the reduction in e-c coupling in muscles subjected to 10 minute glycerol-removal treatments.

3. Conclusions

Figure 52 represents a schematic summary of the possible ultrastructural changes in the T-system of muscles subjected to urea- or glycerol-removal treatments that might account for the effects of these treatments on e-c coupling.

Figure 52A represents a transverse tubule of an untreated muscle in which the marker molecules Horseradish peroxidase and Ferritin freely communicate between extracellular and T-tubular space. The coupling link between excitation and contraction is maintained by calcium-ions (arrows) which are released into the triad junction by depolarization of the tubular membrane. When these calcium ions interact with the membranes of the terminal cisternae they cause a further release of calcium from the SR, which activates the contractile elements.

When muscles are exposed to hypertonic glycerol for 10 minutes and are subsequently reimmersed in an isotonic Ringer's solution the following changes (Figure 52B) will take place: the muscle fibre will rapidly swell and thereafter its volume will gradually return to normal (Krolenko and Adamjan, 1967; Dulhunty and Gage, 1973a). These volume changes represent the initial rapid influx and the subsequent slower efflux of H_2O associated with the outward diffusion of glycerol. The influx of water dilutes the sarcoplasm more rapidly than does the diffusion of glycerol from the transverse tubules, resulting in transient concentration gradients across the membranes of the T-system. As a result water will now be dumped in the tubular lumen causing some swelling of the T-system. The swelling

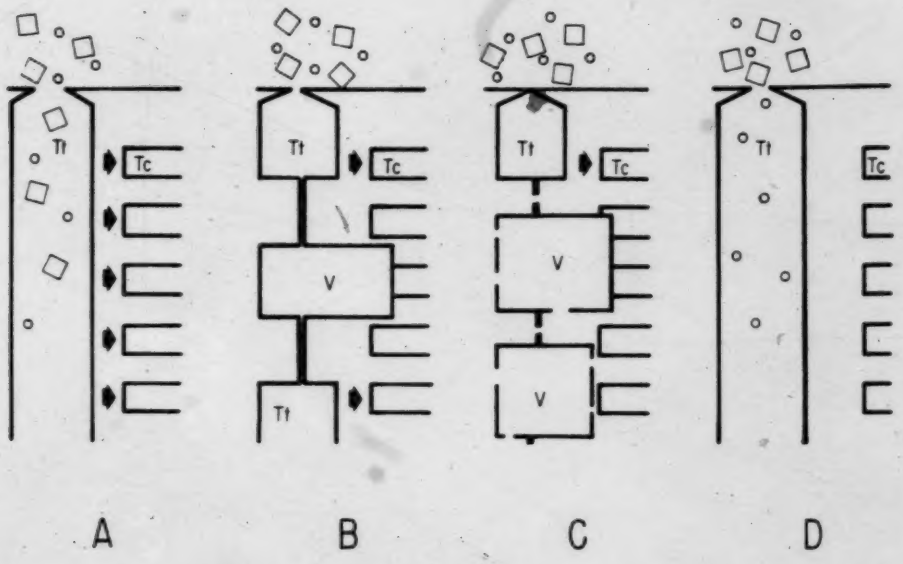

Figure 52. Changes in triad structure following different hypertonic-removal treatments. A schematic drawing of postulated structural changes that may occur in the T-system of muscle fibres subjected to short (10 min) glycerol-removal treatment (B), long (60 min) glycerol-removal treatment (C), urea-removal treatment (D), and untreated muscle fibres (A). Only one junctional face of the triads is depicted and release of trigger-calcium ions across the triadic junction is indicated by arrows. Impairment of the e-c coupling processes by structural rearrangements in the T-system is indicated by the absence of arrows. □, ferritin; o, Horseradish peroxidase; Tt, transverse tubule; Tc, terminal cisterna; V, vacuolated segment of T-tubule. Short glycerol-removal treatment (B) causes vacuolization and narrowing of the T-system. The continuity of the tubular lumen is impaired but the T-tubular membrane is not ruptured. As a result of the osmotically induced structural rearrangements in the T-system the structural integrity in most triads is disturbed and gives rise to a significant reduction in e-c coupling. Long glycerol-removal treatment (C) destroys the continuity of the T-tubular lumen and seals it off from the extracellular space. The effect is more severe than that in (B). The increase in the width of the triad junction in urea-removal treated muscles (D) is the main cause for the interruption of e-c coupling, although some detubulation may also contribute to this effect (see text).

of the long and narrow transverse tubules will cause its membranes to coalesce into large vescicles in some locations, while they may collapse in other parts of the T-system, the overall effect being an interruption of the continuity of the T-tubular lumen (Figure 52B). This explanation is consistent with electromicroscopic observations of irregularly dispersed vacuoles (Figure 45) and measurements of the reduction in the number of intact triads (Table 4B) in muscle fibres subjected to short glycerol-removal treatments. Discontinuity of the T-tubular space is also indicated by the inability of Horseradish peroxidase or Ferritin to enter the T-system. Although the continuity of the tubular lumen may be impaired, the electrical continuity of the T-tubular membranes must be maintained since the normal appearance of the late after potential (Figure 47C) suggests that K^+ ions can accumulate in a restricted extracellular space. If we assume that a series of successive action potentials originating from the surface membrane is able to reach the more centrally located spaces of the T-system, then K^+-ions will accumulate and give rise to a normal LAP response. Because of the breakdown of the tubular continuity one would expect to see a hindrance to the outward diffusion of K^+-ions. The rate of decline of the LAP, which is proportional to the outward K^+ flux (Freygang et al., 1964; Kirsch, 1977), is only slowed down slightly (Figure 48). This reduction is probably insufficient to explain a complete breakdown of the T-tubular continuity, unless a second mechanism for the removal of K^+-ions from the T-tubular lumen is involved. Such a mechanism may in fact be provided by the Na^+, K^+-pump situated in the membranes of muscle fibres and is able to actively transport

K^+-ions across the T-tubular membranes into the sarcoplasm. The possible interaction of the Na^+, K^+-pump with the genesis of the late after potential was not studied in this thesis. Thus in the absence of experimental evidence implicating a second mechanism for the removal of K^+-ions from the T-system I have tentatively concluded that some ion flow must still be maintained through the collapsed regions of the tubular lumen. The partial breakdown of the tubular continuity and consequent structural rearrangements that take place in the triad apparatus (Figure 52B) may impair the normal functioning of the e-c coupling processes at most triadic sites. As a consequence fewer contractile proteins are activated upon electrical stimulation and the twitch response of the muscle is reduced. The severity of the twitch reduction is determined by the proportion of triadic sites in which the e-c coupling processes have been disturbed.

When muscles are exposed to hypertonic glycerol for 60 minutes, and are subsequently reimmersed in an isotonic Ringer's solution, essentially the same osmotic changes take place as those discussed for short glycerol-removal treatments. Some differences between the effects of these treatments were noticed (Figure 52C). A greater degree of disruption of triadic sites was observed (Table 4C) and must have occurred as a result of more prolonged osmotic gradients across the membranes. The longer exposure time to glycerol allowed the muscle fibre to accumulate more glycerol intracellularly and may thus have intensified the degree of osmotic swelling in the T-system. The inability of ferritin or Horseradish peroxidase to penetrate the T-system indicates that continuity of the T-system

with the surface of the fibres is interrupted. The observation that late after potentials or creep potentials could not be elicited in muscles subjected to long (60 min) glycerol-removal treatments may be explained by a disruption of the electrical continuity of the T-tubular membranes. Osmotic stress on the tubular membranes may have become too great causing them to rupture at places. Breakage of the T-tubules near the surface membrane followed by a resealing of the membrane may account for the small drop in resting potentials (Figure 48, inset).

The structural effects of a urea-removal treatment are depicted in Figure 52D. Vacuolization and subsequent detubulation, like that described for glycerol-removal effect, may also contribute to the loss of e-c coupling although the frequency with which these occur is much less than that observed for glycerol-removal treatments. The observation that Horseradish peroxidase enters the T-system indicates that the tubular continuity is preserved in parts of the T-system that are not affected by detubulation. The exclusion of Ferritin from the T-system may be interpreted as a constriction of the transverse tubules at their surface openings. The main structural effect of a urea-removal treatment however is a general increase in the width of the triad junction.

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